Identification of B-cell epitopes on the betanodavirus capsid protein

J Z Costa, A Adams, J E Bron, K D Thompson, W G Starkey and R H Richards

Institute of Aquaculture, University of Stirling, Stirling, UK

Abstract

The pepscan procedure was used to identify betanodavirus B-cell epitopes recognized by neutralizing mouse monoclonal antibodies (MAbs) and serum samples obtained from sea bass, *Dicentrarchus labrax*, naturally infected with betanodavirus. Pepscan was performed with a panel of thirty-four 12-mer synthetic peptides that mimicked the entire betanodavirus capsid protein. Sea bass serum samples reacted strongly with three regions of the capsid protein comprising amino acid residues 1–32, 91–162 and 181–212. The latter region was also recognized by neutralizing MAbs and coincided with a region of high antigenic propensity identified by an antigen prediction algorithm. These data suggest that a region of the betanodavirus capsid protein spanning amino acid residues 181–212 may represent a neutralization domain that could potentially be used to inform the development of nodavirus vaccines and immunodiagnostic reagents.

Keywords: betanodavirus, epitope mapping, luminex, monoclonal antibodies, pepscan, xMAP technology.

Introduction

Within the last 20 years, betanodaviruses have emerged as major pathogens of teleosts, causing disease in several marine and freshwater species (reviewed in Munday, Kwang & Moody 2002). Betanodaviruses are the aetiological agents of viral nervous necrosis (VNN), a disease characterized by vacuolation of the encephalon and retina and the

presence of virus particles within infected neurones. VNN is associated with extensive mortalities that may approach 100% in severe cases. Betanodavirus infection has been reported in more than 35 fish species belonging to 10 different orders including Atlantic cod, *Gadus morhua* L., European sea bass, *Dicentrarchus labrax* (L.), amberjack, *Seriola dumerili* (Risso), groupers, *Epinephelus* spp. and barramundi, *Lates calcarifer* Bloch (Glazebrook, Heasman & Beer 1990; Mori, Nakai, Nagahara, Muroga, Mekuchi & Kanno 1991; Renault, Haffner, Baudin-Laurencin, Breuil & Bonami 1991; Nakai, Nguyen, Nishizawa, Muroga, Arimoto & Ootsuki 1994; Fukuda, Nguyen, Furuhashi & Nakai 1996; Chi, Lo, Kou, Chang, Peng & Chen 1997; Starkey, Ireland, Muir, Jenkins, Roy, Richards & Ferguson 2001; Johnson, Sperker, Leggiero, Groman, Griffiths, Ritchie, Cook & Cusack 2002; Ucko, Colorni & Diamant 2004). Many of the species affected by VNN are of economic importance to the aquaculture industry.

Betanodaviruses are icosahedral viruses with a diameter of 23 nm (Breuil, Bonami, Ppin & Pichot 1991). The betanodavirus genome is bipartite, comprising two single-stranded positive-sense RNA molecules with Mr of 1.01×10^6 Da (RNA1) and 0.49×10^6 Da (RNA2) (Mori, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992). RNA1 encodes a 100-kDa protein representing the viral component of the RNA-dependent RNA polymerase. RNA2 encodes the 42 kDa coat protein precursor (Nagai & Nishizawa 1999). A subgenomic transcript of RNA1, designated as RNA3, is also expressed in infected cells (Somerset & Nerland 2004). Betanodaviruses are genetically diverse, and have been classified into four genotypes based on the nucleotide sequence of the coat protein gene (Nishizawa, Furuhashi, Nagai,

Correspondence Dr J Z Costa, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK (e-mail: janina.costa@stir.ac.uk)

Nakai & Muroga 1997). A betanodavirus with a distinct coat protein nucleotide sequence potentially representing a novel genotype was isolated from sea bass in France (Thiéry, Arnould & Delsert 1999).

The identification of epitopes on viral pathogens is of importance for the rational development of sub-unit vaccines and immunodiagnostic reagents. Relatively few studies have focussed on betanodavirus B-cell epitopes. Nishizawa, Takano & Muroga (1999) identified a putative B-cell epitope located at residues 254–256 of the coat protein, based on differential monoclonal antibody (MAb) binding patterns to recombinant proteins expressed in *Escherichia coli*. However, established mapping procedures have not been used to characterize epitopes in betanodaviruses. In this study, we have used the pepscan procedure (Geysen, Meloen & Barteling 1984) to identify betanodavirus B cells recognized by serum samples from betanodavirus-infected fish, and by anti-betanodavirus mouse MAbs.

Materials and methods

Synthetic peptides

The dragon fish nervous necrosis virus coat protein (Genbank protein access number AAG22496) was used as template for the production of the synthetic peptides, which were produced by Pepscan Systems (Leystad, the Netherlands) using solid phase synthesis and Fmoc chemistry. Peptides were 12 amino acid residues in length with an overlap of two residues and corresponded to the entire betanodavirus coat protein. All peptides contained an N-

terminal amino hexanoic linker. The amino acid sequence of peptides is indicated in Table 1.

Mouse monoclonal antibodies

For production of MAbs, BALB/C mice were immunized with betanodavirus strain MT/01/Sba derived from European sea bass. Mice were immunized twice by intraperitoneal injection with a 1:1 dilution of virus in Titremax® Gold adjuvant (Titremax, Cyt Rx®; Stratech, Cambridge, UK). This was followed by a final intravenous immunization of virus without adjuvant. For each immunization 100 µL of virus (30 µg L⁻¹ stock) was used. The MAbs were produced using standard procedures (Campbell 1984) to promote fusion between SP2/0 cells and mouse splenocytes. Hybridomas were cloned by limiting dilution and selected by ELISA. Four MAbs were used for pepscan analyses: 3B10, 4A12, 4C3 and 5G10. These were purified using Protein-G columns (Hi Trap Protein G HP; GE Healthcare Life Sciences, Amersham, UK). MAb protein concentration was determined with the BCA protein kit (Pierce, Rockford, IL, USA). A MAb produced against *Tetracapsuloides bryosalmonae* was kindly supplied by Dr David Morris (Institute of Aquaculture, University of Stirling, Stirling, UK) for use as a negative control.

Sea bass serum samples

Serum samples were collected from European sea bass naturally infected with betanodavirus. The sea bass were raised in cages in Greece. The presence of antibodies against betanodavirus was assessed by an

Peptide (aa residue)	Amino acid sequence	Peptide (aa residue)	Amino acid sequence
1 (1–12)	MVRKGEKKLAKP	18 (171–182)	SGKEQRLTSPGR
2 (11–22)	KPPTTKAANPQP	19 (181–192)	GRLILLCVGNNT
3 (21–32)	QPRRRANNRRRS	20 (191–202)	NTDVVNVSVLCR
4 (31–42)	RSNRTDAPVSKA	21 (201–212)	CRWSVRLSVPSL
5 (41–52)	KASTVTGFGRG	22 (211–222)	SLETPEETTAPI
6 (51–62)	GTNDVHLSGMSR	23 (212–232)	PIMTQGSLYNDS
7 (61–72)	SRISQAVLPAGT	24 (231–242)	DSLSTNDFKSIL
8 (71–82)	GTGTDGYVVVDA	25 (241–252)	ILLGSTPLDIAP
9 (81–92)	DATIVPDLLPRL	26 (251–262)	APDGAVFQLDRP
10 (91–102)	RLGHAARIFQRY	27 (261–272)	RPLSIDYSLGTG
11 (101–112)	RYAVETLEFEIQ	28 (271–282)	TGDVDRVYWHL
12 (111–122)	IQPMCPANTGGG	29 (281–292)	HLKKFAGNAGTP
13 (121–132)	GGYVAGFLPDPT	30 (291–302)	TPAGWFRWGIWD
14 (131–142)	PTDNDHTFDALQ	31 (301–312)	WDNFNKTFTDGV
15 (141–152)	LQATRGAVVAKW	32 (311–322)	GVAYYSDEQPRQ
16 (151–162)	KWWESRTVRPQY	33 (321–332)	RQILLPVGTVCT
17 (161–172)	QYTRTLLWTSSG	34 (327–338)	VGTVCRTVDSEN

Table 1 Sequences of the synthetic peptides (12-mers) used for epitope mapping. Sequences were derived from dragon fish nervous necrosis virus coat protein (Genbank protein access number AAG22496)

ELISA that utilized cell-culture propagated betanodavirus strain MT/01/Sba as antigen and an anti-European sea bass IgM MAb (Aquatic Diagnostics Ltd, Stirling, UK). Goat anti-mouse IgG conjugated to horseradish peroxidase and tetramethylbenzidine dihydrochloride were used for detection of bound antibodies. The sea bass serum sample used as a negative control was supplied by Dr W. Roy (Machrihanish Environmental Research Laboratory, UK) and was obtained from sea bass farmed in Wales, where nodavirus infection has never been recorded.

Epitope mapping

Synthetic peptides were coupled to polystyrene fluorescent microspheres (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. Peptide-coupled microspheres were blocked with assay buffer [Dulbecco's PBS containing 1% bovine serum albumin (w/v) and 0.02% sodium azide (w/v)] for 30 min at room temperature. The coupling procedure was performed by Pepscan Systems. Filter plates (MultiScreen HTS^{TM} Millipore, Bedford, MA, USA) were blocked with assay buffer (two 30-min incubations at room temperature) to prevent nonspecific antibody binding.

Four types of microsphere, each with a unique spectral address, were used for pepscan analysis. Two thousand of each peptide-coupled microsphere were added per well and the assay buffer was removed using a manifold system (Bio-Rad). To each well, 50 µL MAb or fish serum was added and then incubated overnight at 4 °C with gentle agitation. MAbs were tested at a concentration of 100 µg mL⁻¹ in assay buffer. Fish serum samples were used at a dilution of 1:25 v/v in assay buffer. Peptide-binding antibodies were detected using 50 µL of reporter antibody (25 µg mL⁻¹ in assay buffer). Goat anti-mouse IgG conjugated with phycoerythrin (Molecular Probes, Eugene, OR, USA) was used as a reporter antibody and the incubation with the microsphere–antibody complex was performed at room temperature with gentle agitation for 30 min. For analysis of sea bass serum samples, 50 µL of mouse anti-sea bass IgM MAb (Aquatic Diagnostics Ltd) at a concentration of 20 µg mL⁻¹ was used as a primary antibody. Microspheres were resuspended in 100 µL of assay buffer, and sample fluorescence values were measured on a Bio-Plex^{TM} (Bio-Rad) instrument programmed to analyse a 50 µL sample volume and

to read a minimum of 100 events per each sample analysed. Results are expressed as mean fluorescence intensity (MFI).

Data analysis comprised three steps: (i) Subtraction of the background fluorescence from test sample MFI. Background fluorescence was determined by performing an experimental run in which antibody samples were replaced by assay buffer. (ii) Establishment of cut-off value. The mean and standard deviation of the 50% lowest MFI values were calculated. The cut-off value was then set at 5 × SD above the calculated mean MFI. (iii) To reduce the influence of nonspecific fluorescence when analysing sea bass serum samples, for each peptide the MFI value of a nodavirus-negative serum sample was subtracted from the corresponding MFI value for nodavirus-infected samples. The cut-off value was then established as described in (ii) above.

Hydropathicity analysis of the betanodavirus capsid protein

The hydropathicity profile of the betanodavirus coat protein was analysed in a seven amino acid window using the method developed by Kyte & Doolittle (1982). Analysis was performed using the Pepwindow algorithm (European Molecular Biology Open Software Suite; <http://bioinfo.hku.hk/EMBOSS>).

Prediction of the betanodavirus coat protein structure

The secondary structure of the betanodavirus coat protein was predicted using PredictProtein (McGuffin, Bryson & Jones 2000) (<http://www.predictprotein.org/cgi>).

The CPHmodels 2.0 homology-modelling server (Lund, Nielsen, Lundegaard & Worning 2002) was used to predict the 3D structure of the betanodavirus capsid protein. This algorithm is available on: <http://www.cbs.dtu.dk/services/CPHmodels/> and produced a 3D structure of the capsid protein that incorporated amino acid residues 88–212.

Antigenic prediction

The prediction of potentially antigenic regions of the betanodavirus coat protein was performed using the Kolaskar & Tongaonkar (1990) method. Analysis was performed using the antigenic program (European Molecular Biology Open Software Suite; <http://bioinfo.hku.hk/EMBOSS>).

Results

Epitope mapping with sea bass serum samples

Nine serum samples from betanodavirus-infected sea bass were tested for binding to a panel of synthetic peptides (12-mers) that mimicked the entire betanodavirus capsid protein (Fig. 1). The peptides recognized most strongly by these serum samples were located within three regions of the capsid protein that corresponded to amino acid residues 1–32, 91–162 and 181–222 (Fig. 2). All of the serum samples tested recognized a region of the capsid protein represented by peptides 19–21, corresponding to residues 181–212.

Epitope mapping with mouse monoclonal antibodies

Four neutralizing anti-betanodavirus MAbs were tested for the ability to bind to a panel of synthetic peptides (12-mers) mimicking the betanodavirus capsid protein (Fig. 3). MAb 3B10 recognized peptide 20 (corresponding to capsid protein resi-

Figure 2 Epitope mapping of the betanodavirus coat protein with sea bass serum samples. Combined fluorescence values of nine serum samples against synthetic peptides. X-axis = peptide number; y-axis = fluorescence (MFI; see Materials and methods).

dues 191–202). MAb 4A12 recognized peptides 3, 16 and 19–21 (corresponding to residues 21–32, 151–162 and 181–212). MAb 4C3 recognized peptides 15–16 and 19–20 (corresponding to residues 141–162 and 181–202). MAb 5G10 recognized peptides 16 and 20 (corresponding to residues 161–162 and 191–202).

Figure 1 Epitope mapping of the betanodavirus coat protein with sea bass serum samples. Nine serum samples were studied. X-axis = peptide number; y-axis = fluorescence (MFI; see Materials and methods).

Figure 3 Epitope mapping of the betanodavirus coat protein with mouse monoclonal antibodies. Four monoclonal antibodies were studied, 4C3, 3B10, 4A12, 5G10. X-axis = peptide number; y-axis = fluorescence (MFI; see Materials and methods).

Discussion

The identification of antigenic epitopes on viral pathogens is important for the rational design of vaccines and immunodiagnostic reagents. In this study, we have mapped neutralizing B-cell epitopes on the betanodavirus capsid protein using the pepscan procedure originally developed by Geysen *et al.* (1984). A panel of thirty-four 12-mer peptides mimicking the entire betanodavirus capsid protein was used to map the binding sites of neutralizing anti-betanodavirus MAbs, and serum samples from sea bass naturally infected with betanodavirus.

Serum samples obtained from betanodavirus-infected sea bass strongly recognized three regions of the betanodavirus capsid protein comprising amino acid residues 1–32, 91–162 and 181–212.

The immunogenicity of the N-terminal region of the nodavirus capsid protein has previously been reported by Coeurdacier, Laporte & Pépin (2003). All of the sea bass serum samples recognized a region of the capsid protein spanning residues 181–212. This was also the region of the capsid protein recognized most frequently by neutralizing MAbs. The regions of the capsid protein recognized by MAbs and sea bass serum samples in the present study are distinct from the putative B-cell epitope located at capsid protein residues 254–256 identified by Nishizawa *et al.* (1999). This may be due to antigenic differences between the striped jack nodavirus isolate studied by Nishizawa *et al.* and the sea bass isolate studied in this report.

The region of the betanodavirus capsid spanning amino acid residues 181–212 is strongly hydrophobic (Fig. 4) and contains two potential

Figure 4 Hydropathicity plot of the betanodavirus coat protein showing the position of peptides 19–21. Positive scores indicate hydrophobic residues and negative scores indicate hydrophilic residues.

Amino acid residues 181–212 contain the region of the betanodavirus capsid protein with the greatest antigenic propensity as defined by the antigenic prediction method of Kolaskar & Tongaonkar (1990). Correlation between B-cell epitopes identified in antibody-based mapping studies, and

The results of the present study should be interpreted with caution when applied to the development of nodavirus vaccines. Firstly, the epitopes identified in pepscan analyses represent those recognized by MABs or serum samples with the greatest MFI values. However, these epitopes

could function as immunological decoys, such as those identified on human immunodeficiency virus envelope glycoproteins gp120 and gp41 (Neurath, Strick & Lee 1990) and glycoprotein gp5 of porcine reproductive and respiratory syndrome virus (Fang, Jiang, Xiao, Niu, Zhang & Chen 2006). Secondly, the short 12-mer peptides used for mapping mimic linear epitopes are formed by contiguous stretches of amino acids. Consequently, they cannot detect antibodies that recognize conformational epitopes formed by complex three-dimensional protein folding. Many neutralizing antibodies that recognize viral structural proteins are directed against conformational epitopes (Wolinsky, McCarthy, Allen-Cannady, Moore, Jin, Cao, Lovett & Simmons 1991). However, linear epitopes identified in mapping studies have successfully been used to inform the design of viral vaccines and immuno-diagnostic reagents (Langeveld, Casal, Osterhaus, Cortes, de Swart, Vela, Dalsgaard, Puijk, Schaaper & Melen 1994; Wang, Chang, Walfield, Ye, Shen, Zhang, Lubroth, Chen, Li, Lin, Jong, Yang, Chyr, Kramer & Brown 2001). Thus, the results of the present study highlight regions of the betanodavirus capsid protein that might serve as vaccine components with the objective of inducing protective immune responses. This experimentally testable hypothesis will be the subject of future investigation.

Acknowledgements

Janina Costa was supported by a PhD grant from the Fundação para a Ciência e Tecnologia (FCT), Portugal (SFRH/BD/1269/2000). We gratefully acknowledge help kindly provided by K. F. Muir and J. H. Ireland from the Virology Unit and K. Snedden from the Aquatic Vaccine Unit.

References

- Breuil G., Bonami J.R., Ppin J.-F. & Pichot Y. (1991) Viral infection (picorna-like virus) associated with mass mortalities in hatchery-reared sea-bass (*Dicentrarchus labrax*) larvae and juveniles. *Aquaculture* **97**, 109–116.
- Campbell A.M. (1984) *Monoclonal Antibodies Technology* pp. 256. Elsevier Science Publishers B.V, Oxford, UK.
- Chi S.-C., Lo C.F., Kou G.H., Chang P.S., Peng S.E. & Chen S.N. (1997) Mass mortalities associated with viral nervous necrosis (VNN) disease in two species of hatchery-reared grouper, *Epinephelus fuscogutatus* and *Epinephelus akaara* (Temminck & Schlegel). *Journal of Fish Diseases* **20**, 185–193.
- Coeurdacier J.-L., Laporte F. & Ppin J.-F. (2003) Preliminary approach to find synthetic peptides from nodavirus capsid potentially protective against sea bass viral encephalopathy and retinopathy. *Fish and Shellfish Immunology* **14**, 435–447.
- Fang L., Jiang Y., Xiao S., Niu C., Zhang H. & Chen H. (2006) Enhanced immunogenicity of the modified GP5 of porcine reproductive and respiratory syndrome virus. *Virus Genes* **32**, 5–11.
- Fukuda Y., Nguyen H.D., Furuhashi M. & Nakai T. (1996) Mass mortality of cultured sevenband grouper *Epinephelus septemfasciatus*, associated with viral nervous necrosis. *Fish Pathology* **31**, 165–170.
- Geysen H.M., Meloen R.H. & Barteling S.J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proceedings of the National Academy of Sciences, USA* **81**, 3998–4002.
- Glazebrook J.S., Heasman M.P. & Beer S.W.D. (1990) Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* Bloch. *Journal of Fish Diseases* **13**, 245–249.
- He Y., Zhou Y., Wu H., Kou Z., Liu S. & Jiang S. (2004) Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus. *Journal of Clinical Microbiology* **42**, 5309–5314.
- Johnson S., Sperker S.A., Leggiadro C.T., Groman D.B., Griffiths S.G., Ritchie R., Cook M.D. & Cusack R.R. (2002) Identification and characterization of a piscine neuropathy and nodavirus from juvenile Atlantic cod from the Atlantic coast of North America. *Journal of Aquatic Animal Health* **14**, 124–133.
- Kolaskar A.S. & Tongaonkar P.C. (1990) A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Letters* **276**, 172–174.
- Kyte J. & Doolittle R.F. (1982) A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.
- Langeveld J.P., Casal J.I., Osterhaus A.D., Cortes E., de Swart R., Vela C., Dalsgaard K., Puijk W.C., Schaaper W.M. & Melen R.H. (1994) First peptide vaccine providing protection against viral infection in the target animal: studies of canine parvovirus in dogs. *Journal of Virology* **68**, 4506–4513.
- Lund O., Nielsen M., Lundegaard C. & Wornig P. (2002) X3M a computer program to extract 3D models. In: *CASP5, fifth community wide experiment on the critical assessment of techniques for protein structure prediction. Book of abstracts*. A102. Asilomer, CA, USA.
- McGuffin L.J., Bryson K. & Jones D.T. (2000) The PSIPRED protein structure prediction server. *Bioinformatics* **16**, 404–405.
- Mori K., Nakai T., Nagahara M., Muroga K., Mekuchi T. & Kanno T. (1991) A viral disease in hatchery-reared larvae and juveniles of redspotted grouper. *Gyobyo Kenkyo* **26**, 209–210.
- Mori K., Nakai T., Muroga K., Arimoto M., Mushiaki K. & Furusawa I. (1992) Properties of a new virus belonging to nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology* **187**, 368–371.

- Munday B.L., Kwang J. & Moody N. (2002) Betanodavirus infections of teleost fish: a review. *Journal of Fish Diseases* **25**, 127–142.
- Nagai T. & Nishizawa T. (1999) Sequence on the non-structural protein gene encoded by RNA 1 of striped jack nervous necrosis virus. *Journal of General Virology* **80**, 3019–3022.
- Nakai T., Nguyen H.D., Nishizawa T., Muroga K., Arimoto M. & Ootsuki K. (1994) Occurrence of viral nervous necrosis in kelp grouper and tiger puffer. *Fish Pathology* **29**, 211–212.
- Neurath A.R., Strick N. & Lee E.S. (1990) B cell epitope mapping of human immunodeficiency virus envelope glycoproteins with long (19–36 residue) synthetic peptides. *Journal of General Virology* **71**, 85–95.
- Nishizawa T., Mori K., Furuhashi M., Nakai T., Furusawa I. & Muroga K. (1995) Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish. *Journal of General Virology* **76**, 1563–1569.
- Nishizawa T., Furuhashi M., Nagai T., Nakai T. & Muroga K. (1997) Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Applied and Environmental Microbiology* **63**, 1633–1636.
- Nishizawa T., Takano R. & Muroga K. (1999) Mapping a neutralizing epitope on the coat protein of striped jack nervous necrosis virus. *Journal of General Virology* **80**, 3023–3027.
- Renault T., Haffner P., Baudin-Laurencin F., Breuil G. & Bonami J.R. (1991) Mass mortalities in hatchery-reared sea bass (*Lates calcarifer*) larvae associated with the presence in the brain and retina of virus-like particles. *Bulletin of the European Association of the Fish Pathologists* **11**, 68–73.
- Sommerset I. & Nerland A.H. (2004) Complete sequence of RNA1 and subgenomic RNA3 of Atlantic halibut nodavirus (AHNV). *Diseases of Aquatic Organisms* **58**, 117–125.
- Starkey W.G., Ireland J.H., Muir K.F., Jenkins M.E., Roy W.J., Richards R.H. & Ferguson H.W. (2001) Nodavirus infection in Atlantic cod and Dover sole in the UK. *Veterinary Record* **149**, 179–181.
- Thiéry R., Arnauld C. & Delsert C. (1999) Two isolates of sea bass, *Dicentrarchus labrax* L., nervous necrosis virus with distinct genomes. *Journal of Fish Diseases* **22**, 201–207.
- Ucko M., Colorni A. & Diamant A. (2004) Nodavirus infections in Israeli mariculture. *Journal of Fish Diseases* **27**, 459–469.
- Wang C.Y., Chang T.Y., Walfield A.M., Ye J., Shen M., Zhang M.L., Lubroth J.L., Chen S.P., Li M.C., Lin Y.L., Jong M.W., Yang P.G., Chyr N., Kramer E. & Brown F. (2001) Synthetic peptide based vaccine and diagnostic system for effective control of foot and mouth disease. *Biologicals* **29**, 221–228.
- Wolinsky J.S., McCarthy M., Allen-Cannady O., Moore W.T., Jin R., Cao S.N., Lovett A. & Simmons D. (1991) Monoclonal antibody-defined epitope map of expressed rubella virus protein domains. *Journal of Virology* **65**, 3986–3994.

Received: 22 August 2006

Revision received: 12 December 2006

Accepted: 13 December 2006