DETECTION OF INFECTIOUS BURSAL DISEASE VIRUS BY ELISA USING AN ANTIPEPTIDE ANTIBODY RAISED AGAINST VP3 REGION

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Summary. – Antigenic determinant analysis was carried out on VP3, one of major immunogenic proteins of Infectious bursal disease virus (IBDV) using computer algorithms. Altogether 17 peptides were synthesized for predicted putative regions and were tested for their reactivity with IBDV-positive polyclonal sera as well as with antisera to other common avian viruses to confirm specificity and to rule out cross reactivity. Of 17 peptides tested, three were selected and synthesized in multiple antigenic peptide (MAP) format. The immunization of rabbits with the three MAPs resulted in high humoral immune response. The purified antipeptide antibodies were screened against native IBDV antigen and the respective titers were determined. Out of the three antisera to MAPs that raised against the MAP3, spanning the amino acids (aa) 974–995 region on the VP3 protein had a very high titer (2048) and reacted specifically with IBDV. Thus, the antiserum to MAP3 detected native virus in enzyme-linked immunosorbent assay (ELISA), revealing the presence of a potential antigenic determinant on the C-terminus of the protein. This study proved that an antipeptide antibody could be used as a safe and specific tool for the diagnosis of IBD in chickens.

Key words: antipeptide antibodies; Infectious bursal disease virus; multiple antigenic peptide; antigenic determinants; diagnosis

Introduction

IBD, also known as Gumboro disease (Cosgrove, 1962), is a major viral immunosuppressive disease of young chickens. Although the mortality pattern is inconsistent, economic losses are due to secondary microbial infections (Hirai *et al*., 1974; Saif, 1991). IBDV (the species *Infectious bursal disease virus*, the genus *Avibiranvirus,* the family *Birnaviridae*) (van Regenmortel *et al*., 2000) is a non-enveloped icosahedral virus of 60 nm in diameter (Hirai *et al*., 1974), consisting of two segments (A and B) of dsRNA (Muller *et al*., 1979). The smaller segment B (2.8 kb) encodes VP1, the putative dsRNA polymerase (Azad *et al*., 1985), while the larger segment A (3.3 kb) encodes a 110 K polyprotein N-VP2-VP4-VP3-C (Hudson *et al*., 1986) and a 17 K VP5, speculated to induce apoptosis in cell cultures (Tanimura and Sharma, 1998). The precursor polyprotein is processed into mature viral proteins VP2 (37–40 K), VP3 (32–35 K) and VP4 (24 K) (Hudson *et al*., 1986). Among these, VP2 and VP3 are major structural proteins of the virion, forming the proteinaceous shell. VP3 contains serotype, group-specific and cross-reactive antigenic sites

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Abbreviations: aa = amino acid; AGPT = agar gel precipitation test; ELISA = enzyme-linked immunosorbent assay; FAT = fluorescent antibody technique; FAV= Fowl adenovirus; FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant; FMOC = 9-fluorenyl-methoxy-carbonyl; gly-gly = glycine-glycine; HBTU = 2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate; IBD = infectious bursal disease; IBDV = IBD virus; MAP = multiple antigenic peptide; PAM = 4-hydroxy-methyl-phenyl-acetamido-methyl; PBS = phosphate-buffered saline; RDV = Ranikhet disease virus; SNT = serum neutralization test

(Mahardika and Becht, 1995); a linear epitope has been localized at the C-terminus of this protein spanning aa 908–1012 on VP3 (Yamaguchi *et al*., 1996).

The diagnosis of IBD relies mainly on conventional techniques like virus isolation, agar gel precipitation test (AGPT), counter-immuno-electrophoresis, histopathology, indirect immuno-peroxidase test, indirect ELISA, serum neutralization test (SNT), fluorescent antibody technique (FAT), and latex agglutination test (Hirai *et al*., 1974; Durojaiye *et al*., 1985; Kumar and Rao, 1992; Sah *et al*., 1995; Marquardt *et al*., 1980; Howie and Thorsen, 1981; Jackwood and Saif, 1987; Nachimuthu *et al*., 1995). However, these tests are disadvantageous, namely they are time consuming, poorly sensitive and non-specific. Modern molecular techniques like hybridizations using nucleic acid probes and RT-PCR are very useful for the diagnosis of IBD (Kataria *et al*., 2001). However, synthetic peptides and antisera/antibodies against them offer an approach to development of a safe and chemically defined method for detection of the virus in bursal samples.

Synthetic peptides have turned out to be good therapeutics, immunogens and diagnostic tools. They often mimic natural regions occurring within viral antigens. Prediction of antigenic determinants on immunogenic proteins of viruses and testing of the resulting peptides offer a simple means of developing virus detection methods.

In this study, we employed an approach of this kind in developing a method for detection of the IBD in poultry. The mapping of epitopes of several viral antigens using synthetic peptides and monoclonal antibodies has been done by Geysen *et al*. (1984). Similarly, synthetic peptides have been used for detection of antibodies to many viruses such as HIV-1 (Lombardi *et al*., 1983), Hepatitis C virus (Rosa *et al*., 1995), Epstein-Barr virus (van Grunsven *et al*., 1994), Equine infectious anemia virus (Ball *et al*., 1992) as well as for differential diagnosis of HIV-1 and HIV-2 (Gnann *et al*., 1987).

Keeping all this knowledge in view, the present study was attempted to identify a specific antigenic determinant on VP3 of IBDV and to develop a method based on an antibody against a peptide for diagnosis of IBDV infection in poultry.

Materials and Methods

Biologicals and immunologicals. The virulent Indian Cari strain of IBDV, antisera to IBDV, antisera to Ranikhet disease virus (RDV), antisera to Fowl adenovirus (FAV), and IBDV-negative sera were obtained from the Division of Avian Diseases, Indian Veterinary Research Institute.

Animals. Six-month-old New Zealand white rabbits of either sex from the Laboratory of Animal Resources, Indian Veterinary Research Institute, were used for immunization.

Prediction of antigenic determinants. Sequence data for various strains of IBDV were obtained from the EMBL GeneBank database. The strains with their Acc. Nos. were as follows: strain 002-73, P08364; strain Cu-1, P15480; strain Edgar, A33255; GZ29112, AF051837; strain OKYM, D49706; strain PBG-98, P25220; strain STC, P22351; strain 52/70, P25219; and strain UK661, X92761 of IBDV serotype 1 and strain OH, P27276 of serotype 2.

The sequences were used in the Laser Gene Software Package of DNA-STAR Program for the algorithmic prediction of secondary structures and antigenic determinants on VP3 of IBDV. Comparative analyses for the aforementioned strains were performed and consensus sequences were chosen for peptide synthesis.

Synthesis of bead-bound peptides for selected sequences. Peptides for selected regions of VP3 were synthesized on 4-hydroxymethyl-phenyl-acetamido-methyl resin (PAM resin) beads.

A Glycine-Glycine (Gly-Gly) spacer was attached to the PAM resin beads and 1 mg of beads for the synthesis of each peptide was used. The amino acid sequences used for synthesis of the peptides corresponding to the VP3 region are given in Table 1. The peptides were synthesized by solid-phase peptide synthesis (Merrifield, 1963) by employing the 9-fluorenyl-methoxy-carbonyl (FMOC) chemistry with HBTU (2-(1H-benzotriazole-1-yl)-oxy-1,1,3,3-tetramethyluronium hexafluorophosphate) coupling. The bead-bound peptides were dried and stored at 4°C. The synthesis working with 100% yields was checked by simultaneous random peptide synthesis, by hydrolyzing the peptide using 6 N HCl, and by checking the resulting amino acids by thin layer chromatography and high performance liquid chromatography.

ELISA for screening of synthetic bead-bound peptides. A modification of liquid phase ELISA (Syu and Kahan, 1991) was employed. The bead-bound peptides were treated with a blocking buffer (10% (v/v) fetal bovine serum (FBS), l0% (w/v) bovine serum albumin, 0.05% Tweeen 20, all in phosphate-buffered saline (PBS) pH 7.2) for 2 hrs at 37°C. A hundred µl of a serum (1:50 dilution) was added and incubated in a microcentrifuge tube for 1 hr at 37°C. The unreacted antibodies were removed by washing the beads five times with the PBS-T buffer (0.05% Tween 20 in PBS) and by pelleting the beads by centrifugation at 4,000 x g for 5 mins and then the supernatant was removed. The beads were incubated with a rabbit anti-chicken IgG conjugated with horse radish peroxidase, diluted 1:20, 000 in the blocking buffer, for 2 hrs at 37°C. After stringent washing, 100 µl of a substrate solution (7 mg of ortho-phenylene-diamine and 8 µl of H_2O_2 in 10 ml of citric-acid phosphate buffer pH 5.0) was added and kept at 37°C for 15 mins in dark for color development. Then from each tube 100 µl of the supernatant was transferred to the wells of a 96-well ELISA plate and the reaction was stopped by the addition 100 µl of 1 mol/l H_2SO_4. A_{492} was read in an ELISA reader.

Regeneration of bead-bound peptides. After each ELISA the peptides were regenerated by breaking the peptide-antibody complexes by soaking the beads twice in 300 ml of an urea buffer (8 mol/l urea, 0.1 % SDS, and 0.1% 2-mercaptoethanol) for 2 x 10 mins followed by 5-fold washing in PBS-T. In this way the bead-bound peptides were regenerated and reused for more than 50 times.

Synthesis of MAPs. Three MAPs were synthesized by direct FMOC chemistry with HBTU coupling (Tam, 1988). Alanine was attached to the Wang resin followed by lysine branching to form

Table 1. Sequences of peptides synthesized for VP3 region of IBDV

Serial No.	Region on VP3	Length (No. of aa)	Amino acid sequence
1	p722–735	14	IKRFPHNPRDWDRL
2	p745–754	10	PNAGRQYDLA
3	p758–768	11	SEFKETPELES
4	p806–817	12	ALSDPNAHRMRN
5	p824–837	14	QAGSKSQRAKYGTA
6	p841–849	9	VEARGPTPE
7	p852–865	14	QREKDTRISKKMET
8	p881-890	10	HRGPSPGQLK
9	p893–904	12	QNTREIPDPNED
10	p907–921	15	DYVHAEKSRLASEGQ
11	p932–941	10	APGQAEPPQA
12	p951–965	15	EVNHGRGPNQEQMKD
13	p971–981	11	MEMKHRNPRRA
14	p974–995	22	KHRNPRRAPPKPKPKPNVPTQR
15	p983–995	13	PKPKPKPNVPTQR
16	p1004–1013A	10	IRAVSDEDLE
17	p1004–1013T	10	IRTVSDEDLE
18	pRandom	10	PSARAVANAN

Peptide Nos. 16 and 17 differ by a single amino acid at the position 1006.

eight dendritic arms of core matrix over which each peptide sequence was synthesized. The MAPs were cleaved from the resin with 95% trifluoroacetic acid in water, dried and stored at -70°C. The purity of the resulting MAPs was checked by reverse phase high performance liquid chromatography using a RPC-250 T column.

Production of antisera to MAPs in rabbits. The antisera were produced in New Zealand white rabbits according to McLean *et al*. (1991) with necessary modifications. Rabbits were injected intramuscularly with each MAP emulsified in equal volume of Freund's complete adjuvant (FCA) in the first injection and then in Freund's incomplete adjuvant (FIA) (Sigma) in subsequent injections. Rabbits were injected on days 0, 10, 30, and 40 with 75, 100, 125 and 150 µg, respectively, of each MAP and bled on days 0, 7, 17, 37, and 50. A final booster dose of 200 µg of each MAP in FIA was administered on day 90 and bled on day 97 post immunization. The antisera were used in ELISA for screening of bursal samples. For each MAP four rabbits were immunized and the serum from the rabbit showing the highest titer was used for titration of the antisera to MAPs. In total 16 rabbits were immunized: four rabbits for each MAP, and four rabbits inoculated with PBS served as controls.

Purification of antipeptide antibodies by DEAE-Sephadex chromatography. The antipeptide antibodies were purified from the anitsera by a simple one-step ion-exchange chromatography according to Page and Thorpe (1996). Briefly, micropipette tips (1 ml capacity) were packed with 500 mg of DEAE-Sephadex in sterile water. The column was equilibrated with a sodium phosphate buffer pH 6.3. Then 1 ml of a serum sample was overlaid on top of the column and the eluate was saved. Then the column was thoroughly eluted with 4x1 ml of the buffer mentioned above and all the eluates were pooled and used in ELISA.

Virus cultivation and purification. IBDV was propagated in primary chicken embryo fibroblast (CEF) cultures. CEF cultures were grown in Glasgow Minimum Essential Medium (GMEM) supplemented with 10% of newborn calf serum. A stock virus was diluted 1:100 and left to adsorb onto a confluent cell monolayer in Roux flasks for 45 mins. Then a maintenance medium (GMEM supplemented with 2% of newborn calf serum) was added and the cultures were incubated at 37°C. The cultures were harvested at 72 to 96 hrs post infection (p.i.), when typical cytopathic changes like rounding, focal necrosis and detachment of cells from the surface were observed in 80–90% of cells. The virus was purified according to Cruz-Coy *et al*. (1993) with necessary modifications. Cultures in Roux flasks were subjected to 3 cycles of freezing and thawing and the cell debris were pelleted by a centrifugation at 3,000 x g for 20 mins. Virus in the supernatant was pelleted at 100,000 x g for 2 hrs in a Sorvall ultracentrifuge at 4°C. The virus in the pellet was resuspended in 1 ml of TNE buffer (50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, and 10 mmol/l EDTA) and pelleted at 120,000 x g for 3 hrs at 4°C on a cushion of discontinuous 30%–60% sucrose gradient. The opaque band at interface containing the virus was collected. Sucrose was removed by diluting the virus at least five times in TNE buffer and by pelleting again at 100,000 x g for 2 hrs at 4°C. The final virus pellet was resuspended in TNE buffer pH 8.0 and stored at -20°C until use.

Titration of antipeptide antibodies were determined by indirect ELISA using 2 ng of a MAP for coating each well. Similarly, the reactivity of the antipeptide antisera was evaluated using 0.8 µg of IBDV whole antigen in a separate experiment.

Mean A_{492} values higher than the 2-fold of A_{492} value of the negative control were considered positive. Titers were expressed as reciprocals of the highest dilutions of sera showing positive reaction.

Evaluation of bursal samples. Twenty clinical bursal samples, processed as 10% suspensions in PBS, were tested by AGPT and indirect ELISA.

Results and Discussion

Identification of antigenic determinants on VP3 protein of IBDV

Using the Laser Gene Software of DNA STAR Program and computer modeling the prediction of antigenic determinants on VP3 protein (aa 724–1012) of IBDV was performed and the sequences are shown in Table1. The abovementioned Program predicts antigenic determinants according to a score generated on the basis of secondary structure (Chou and Fasman, 1978; Garnier *et al.,* 1978), hydrophilicity (Kyte and Doolittle, 1982), flexibility (Karplus and Schultz, 1985), surface probability, and antigenic index (Jameson and Wolf, 1988). The used algorithm predicts only the likelihood of a potential epitope and hence requires actual verification.

Of the two serotypes of IBDV, the serotype 1 is pathogenic to chicken, while the serotype 2 is apathogenic to chicken and was isolated from turkeys (Ismail *et al*., 1988). The obtained sequences were aligned and analyzed for the strains 9 and 1 of the serotyopes 1 and 2, respectively. The sequences conserved among the serotype 1 strains and variable for the serotype 2 strain OH were used for prediction of antigenic determinants. In this way 17 probable antigenic determinants were predicted.

Synthesis of bead-bound peptide analogs

A total of 17 peptides for VP3 and one control peptide with a random sequence of PSARAVANAN (10-mer) were synthesized. The location of potential regions and the amino acid sequences and the length of the peptides are depicted in Table 1. The analysis revealed 17 probable regions within VP3, the length ranging from 9 to 22 amino acids. The peptides were successfully synthesized by solid phase peptide synthesis using PAM beads (1 mg per peptide). In order to maintain a desired gap between the peptide epitope and bead surface to facilitate the antibody reaction and to avoid interference in ELISA, a Gly-Gly spacer was linked onto the PAM beads, which was found to be the optimal spacer as reported earlier (Yu and Chu, 1997). The results indicated that the PAM beads with Gly-Gly helped in the efficient projection of the peptide epitope to the antibody without any hindrance in ELISA, as glycine is the only amino acid without any side chain. It was evident from this study that 1 mg of PAM beads were sufficient for each peptide synthesis and subsequent screening.

Screening of peptides

The reactivity of peptides screened by liquid phase indirect ELISA with IBDV-positive and IBDV-negative, RDV-positive and FAV type4-positive sera are shown in Table 2. Mean A_{492} values higher than the 2-fold of A_{492} value of the control peptide (pRandom) were considered positive. It is evident that the peptides reacted specifically with anti-IBDV antibodies and that there were no cross reactions with antibodies to other avian viruses such as RDV and FAV at the same serum dilution (1:50). The peptides were repeatedly regenerated (50 times) by breaking the antigen-antibody complexes with the urea buffer and reused without any change in their activity.

This study established that the peptides of VP3 of IBDV were specific to anti-IBDV antibodies and could help in detecting anti-IBDV antibodies in chicken sera in the presence of other antibodies. The advantage of this method is the possible reuse of peptides in at least 50 tests.

Three specific peptides and a random peptide were negative with IBDV antibodies in ELISA and were excluded. The first three peptides that displayed a maximum absorbance with a positive anti-IBDV antiserum and a negligible absorbance with RDV and FAV antisera were selected for use in further experiments.

Production of anti-peptide antibodies

The amino acid sequences of MAPs synthesized for highly reactive VP3 are shown in Table 3. Three MAPs were synthesized and used for immunization of rabbits. The ELISA titers of anti-MAP antisera are depicted in Table 4. The antisera raised against MAP1 and MAP2 showed very low titers, namely 64 and 128,respectively, while the anti-MAP3 antiserum had a titer of 2048 on the 50^{th} and 97^{th} days post immunization with both MAP3 and native antigen.

This clearly indicated that the MAP3 anti-peptide antibodies reacted specifically with IBDV in ELISA, signifying the presence of a potent antigenic determinant, which could help in diagnosis of IBD at an early stage of infection. Furthermore, the fact that the MAP3 anti-peptide antibodies reacted with the native antigen and that they were able to recognize the cognate sequence present in the native virus, is an interesting finding of this study. Similarly, these anitsera were titrated using the native antigen (Table 4). The failure of the anti-peptide antibodies to MAP1 and MAP2 to recognize the native virus as such in ELISA could be either due to the absence of the respective antigenic determinants or to the dissimilarity in the epitope conformation.

When 2 and 4 copies of each of the three MAPs were used for immunization they failed to elicit an immune response. The resulting antisera were of very low titers (<4). Therefore, it was concluded that a minimum of 8 copies of a MAP is required for induction of a significant antibody response and that the use of a carrier protein is not necessary. Thus MAP3 antisera could be effectively utilized for the detection of IBDV in enzootic and mixed infections.

Table 2. ELISA screening results of IBDV VP3-specific peptides with different sera samples

Region on VP3	IBDV-positive	IBDV-negative	RDV-positive	FAV-positive
	A_{492}			
p974–995[a]	0.652	0.071	0.048	0.051
p722–735[a]	0.636	0.046	0.054	0.098
p841–849[a]	0.617	0.056	0.009	0.063
p806–817	0.590	0.138	0.005	0.056
p983–995	0.576	0.008	0.008	0.080
p971–981	0.557	0.108	0.008	0.072
p881–890	0.550	0.158	0.004	0.071
p745–754	0.538	0.106	0.018	0.096
p1004–1013T	0.531	0.172	0.006	0.025
p951–965	0.505	0.005	0.006	0.025
p932–941	0.484	0.013	0.005	0.081
p907–921	0.468	0.151	0.004	0.065
p852–865	0.467	0.186	0.007	0.091
p893–904	0.458	0.055	0.004	0.044
[b]p1004–1013A	0.390	0.068	0.006	0.049
[b]p758–768	0.353	0.088	0.016	0.056
[b]pRandom	0.201	0.125	0.146	0.103
[b]p824–837	0.187	0.148	0.015	0.029

[a]Peptides showing strong reaction with anti-IBDV antibodies in ELISA.
[b]Peptides showing negative reaction with anti-IBDV antibodies in ELISA.
All the sera are polyclonal and used at 1:50 dilution.

Table 3. Amino acid sequences of MAPs synthesized for the VP3 region of IBDV

MAP	Region on IBDV	Length (No. of aa)	No. of copies of each peptide	Sequence	Calculated M_r
MAP1	722-735	14	8	IKRFPHNPRDWDRL	17767
MAP2	841-849	9	8	VEARGPTPE	9895
MAP3	974-995	22	8	KHRNPRRAPPKPKPKPNVPTQR	24911

Table 4. Titers of anti-peptide antisera raised against the three MAPs in ELISA

Day post immunization	ELISA titers					
	MAP1[a]	Virus[b]	MAP2[a]	Virus[b]	MAP3[a]	Virus[b]
0	0	0	0	0	0	0
7	2	0	8	0	16	16
17	4	0	32	0	64	64
37	16	0	64	0	512	512
50	64	0	128	0	2048	2048
97	64	0	128	0	2048	2048

[a]Titers of anti-peptide antisera in ELISA using respective MAP as antigen.
[b]Titers of anti-peptide antisera in ELISA using IBDV as antigen.
A booster dose of 200 µg of each MAP in Freund's incomplete adjuvant was administered by i. m. injection on day 90 and bled on day 7 after the booster. Anti-peptide antisera raised against MAP1 and MAP2 failed to bind native virus coated in ELISA and gave zero titer, while the anti-peptide antiserum against MAP3 (p974–995), showing positive reaction in the ELISA plate coated with native virus and their respective titers, are shown.

In this study, for field evaluation, 20 bursal samples were screened by conventional AGPT and ELISA with the anti-MAP3 antiserum for its efficacy in detecting whole viral antigen. The ELISA using the anti-peptide antibody and anti-IBDV hyper immune sera performed equally well in detecting the virus in bursal samples with a 90% positivity and was more sensitive than the AGPT, which gave only a 70% positivity.

In conclusion, it is suggested that the region of aa 974–995 on VP3 is a potential antigenic determinant specific to

Table 5. Comparison of sensitivity of AGPT, standard ELISA and anti-peptide antibody ELISA in detecting the virus in crude bursal extracts using anti-peptide antibody against MAP3 and IBDV-positive sera

No. of bursal samples tested	Positivity (%)		
	AGPT	Standard ELISA	Anti-peptide antibody ELISA
20	14 (70%)	18 (90%)	18 (90%)

AGPT was performed using standard anti-IBDV-positive sera and tested bursal samples. Standard ELISA with IBDV-positive sera and anti-peptide antibody ELISA with the antisera raised against MAP3 (VP3 region of IBDV) were of the same accuracy and sensitivity. Values in parentheses indicate the percentage of positivity.

IBDV and that the anti-peptide antibodies using the same peptide in MAP format for the same region could detect the virus in clinical bursal samples. Prospectively, these products could be linked to any biosensor molecule for the development of a diagnostic kit for field use and for the development of a synthetic peptide vaccine.

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References

Azad AA, Barrett SA, Fahey KJ (1985): The characterization and molecular cloning of the ds RNA genome of an Australian strain of infectious bursal disease virus. *Virology* **143**, 35–44.

Ball JM, Rushlow KE, Issel CJ, Montelaro RC (1992): Detailed mapping of the antigenicity of the surface unit glycoprotein of equine infectious anaemia virus by using synthetic peptide strategies. *J. Virol.* **66**, 732–742.

Chou PY, Fasman GD (1978): Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**, 145–148.

Cosgrove AS (1962): An apparently new disease of chickens-avian nephrosis. *Avian Dis.* **9**, 385–389.

Cruz-Coy JS, Giambrone JJ, Panangala VS (1993): Production and characterization of monoclonal antibodies against a variant infectious bursal disease virus. *Avian Dis.* **37**, 406–411.

Durojaiye OA, Adene DF, Owoade, AA (1985): Counter immunoelectroosmophoresis in the diagnosis of infectious bursal disease of poultry. *Trop. Anim. Health Prod.* **17**, 225–229.

Garnier J, Osguthorpe DJ, Robson B (1978): Analysis of the accuracy and implications of simple methods for predicting the structure of globular proteins. *J. Mol. Biol*. **20**, 97–120.

Geysen HM, Meloen RH, Barteling SJ (1984): Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. USA* **81**, 3998–4002.

Gnann JWJr, McCormic JB, Mitchell S, Nelson JA, Oldstone MBA (1987): Synthetic peptide immunoassay distinguishes HIV type 1 and HIV type 2 infections. *Science* **237**, 1346–1349.

Hirai K, Shimakura S, Kawamoto E, Taguchi F, Kim ST, Chang CN, Ititani Y (1974): The immunodepressive effect of infectious bursal disease virus in chickens. *Avian Dis.* **18**, 50–57.

Howie R, Thorsen J (1981): An enzyme-linked immunosorbent assay (ELISA) for infectious bursal disease virus. *Can. J. Comp. Med*. **45**, 51–55.

Hudson PJ, Mckern NM, Power BE, Azad AA (1986): Genomic structure of the large RNA segment of IBDV. *Nucleic Acids Res.* **14**, 5001–5012.

Ismail NM, Saif YM, Moorhead PD (1988): Lack of pathogenicity of five serotype2 infectious bursal disease viruses. *Avian Dis*. **32**, 757–759.

Jackwood DH, Saif YM (1987): Antigenic diversity of infectious bursal disease viruses. *Avian Dis.* **31**, 766–770.

Jameson BA, Wolf H (1988): The antigenic index: a novel algorithm for predicting antigenic determinants. *Comput. Appl. Biosci.* **4**, 181–186.

Karplus PA, Schultz GE (1985): Prediction of chain flexibility in proteins. *Naturwissenschaften*. **72**, 212–222.

Kataria RS, Tiwari AK, Nanthakumar T, Goswami PP (2001): One-step RT-PCR for the detection of infectious bursal disease virus in clinical samples. *Vet. Res. Commun.* **25**, 429–436.

Kumar A, Rao AT (1992): An indirect immunoperoxidase method for diagnosis of infectious bursal disease. *Indian Vet. J*. **69**, 176–177.

Kyte J, Doolittle RF (1982): A simple method for displaying hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132.

Lombardi V, Placido R, Scarlatt G, Romiti ML, Mattei M, Mariani F, Poccia F, Rossi P, Calizzi V (1983): Epitope specificity-antibody dependent cell mediated cytotoxicity and neutralizing activity of antibodies to HIV Type 1 in autoimmune MRL/Ipr mice. *J. Infect. Dis* **167**, 1267–1273.

Mahardika GNK, Becht H (1995): Mapping of cross-reacting and serotype specific epitopes on the VP3 structural protein of IBDV. *Arch. Virol.* **140**, 765–774.

Marquardt WM, Johnson RB, Odenwald WF, Schlotthober BA (1980): An indirect enzyme-linked immunosorbent assay (ELISA) for measuring antibodies in chicken infected with infectious bursal disease virus. *Avian Dis.* **24**, 375–385.

McLean GW, Owsianka AM, Subak-Sharpe JH, Marsden HS (1991): Generation of anti-peptide and anti-protein sera: Effect of peptide presentation on immunogenicity. *J. Immonol. Methods* **137**, 149–157.

Merifield RB (1963): Solid phase peptide synthesis I. The synthesis of tetrapeptide. *J. Am. Chem. Soc.* **85**, 2149–2154.

Muller H, Scholtissek C, Becht H (1979): The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. *J. Virol.* **31**, 584–589.

Nachimuthu K, Raj GD, Thangavelu A, Venkatesan RA (1995): Reverse passive haemagglutination test in the diagnosis of infectious bursal disease. *Trop. Anim. Health Prod.* **27**, 43–46.

Page M, Thorpe R (1996): JM Walker (Eds): *The Protein Protocols Handbook*. Humana Press Inc. Totowa, NJ, USA, pp. 725–726.

Rosa C, Osborne S, Garetto F, Griva S, Rivella A, Calabresi G, Guaschino R, Bonelli F (1995): Epitope mapping of the NS4 and NS5 gene products of hepatitis C virus and the use of a chimeric NS4-NS5 synthetic peptide for serodiagnosis. *J. Virol. Methods* **55**, 219–232.

Sah RL, Kataria JM, Arya SC, Verma KC (1995): Ourbreaks of acute IBD causing high mortality in chicken. *Indian J. Comp. Immun. Microbial. Infect. Dis.* **16**, 7–13.

Saif YM (1991): Immunosuppression induced by infectious bursal disease virus. *Vet. Immunol. Immunopathol.* **30**, 45–50.

Syu WJ, Kahan L (1991): Characterization of antigenic structures by mapping on resin-bound epitope analogs. *Methods Enzymol.* **203**, 292–300.

Tam JP (1988): Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA* **85**, 5409–5413.

Tanimura N, Sharma JM (1998): In-situ apoptosis in chickens infected with infectious bursal disease virus. *J. Comp. Pathol.* **118**, 15–27.

van Grunsven WM, Spaan WJ, Middeldorp JM (1994): Localization and diagnostic application of immunodominant domains of the BFRF-3-encoded Epstein-Barr virus capsid protein. *J. Infect. Dis.* **170**, 13–19.

van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego-San Francisco-New York-Boston-London-Sydney-Tokyo.

Yamaguchi T, Iwata K, Kobayashi M, Ogawa M, Fukushi H, Hirai K (1996): Epitope mapping of capsid proteins VP2 and VP3 of IBDV. *Arch. Virol.* **141**, 1493–1507.

Yu Z, Chu Y (1997): Combinatorial epitope search: pitfalls of library design. *Bioorg. Med. Chem. Lett.* **7**, 95–98.