



**UNIVERSITI PUTRA MALAYSIA**

**MOLECULAR CHARACTERISATION AND PATHOGENICITY OF  
INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN IRAN**

**MOHAMMAD ALI BAHMANINEJAD**

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**By**

**MOHAMMAD ALI BAHMANINEJAD**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

**September 2004**



## **DEDICATION**

**DEDICATED WITH LOVE AND APPRETIATION TO  
MY WIFE AND CHILDREN  
FOR THEIR ETERNAL LOVE AND KINDNESS**

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OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN  
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**By**

**MOHAMMAD ALI BAHMANINEJAD**

**April 2004**

**Chairman: Associate Professor Dr. Mohd Hair-Bejo, Ph.D.**

**Faculty: Veterinary Medicine**

Infectious bursal disease (IBD) is one of the most important diseases among the poultry industries in Iran. Ten IBD virus (IBDV) isolates were obtained from IBD field outbreaks. These isolates were from the vaccinated and non IBD vaccinated broiler and layer flocks in Iran and were designated as IR197, IR297, IR198, IR298, IR398, IR199, IR299, IR399, IR499 and IR599.

These isolates showed clinical signs, mortality, gross and histological lesions in commercial chickens that were typical of IBD during field outbreaks. The Iranian IBDV isolates were isolated and identified by conventional methods including agar gel precipitation test (AGPT), egg inoculation, chicken embryo fibroblast (CEF) cell culture, transmission electron microscopy (TEM), and immunoperoxidase staining (IPS) as IBDV. These isolates could not propagate onto CEF cell culture except IR298 isolate, which produced cytopathic effect (CPE) in cell culture. IR298 and IR499 isolates were

inoculated into 28-day-old specific pathogen free (SPF) chicken; IR298 isolate did not produce any mortality and the gross lesions in SPF chickens but the bursa of Fabricius was atrophied, whereas IR499 isolate induced 70% mortality, gross and histological lesions which were typical of very virulent (vv) vvIBDV.

All isolates were further characterised by molecular techniques based on RT-PCR-RFLP and nested PCR. The hypervariable region of VP2 gene were amplified and sequenced. The phylogenetic tree was constructed based on aligned sequences of the Iranian isolates and published IBDV strains. All the isolates had the characteristics of vvIBDV strain similar to the earlier reports, except IR298 isolate showed the characteristics of vaccine strain. The phylogenetic tree of the isolates showed that all isolates except IR298 belongs to vvIBDV subgroups of serotype 1. Isolates IR197, IR299, IR399, IR398, IR499 and IR599 formed the distinct sub branch within the subgroup of vvIBDV strain. IR298 isolate was located within the subgroup of the vaccine strain. The origin of these isolates could be similar to the vvIBDV strains isolated in Europe, Japan and Hong Kong, whilst IR298 isolate could be similar to the Chinese and Egyptian classical as well as vaccine strains.

The IR499 isolate ( $10^{6.7}$  EID<sub>50</sub>) was inoculated in 28-day-old SPF chickens via oral route to determine the response of gut-associated lymphoid tissues (GALT) to vvIBDV isolated in Iran. The GALT were lymphoid cell aggregations at the oesophagus and proventriculus junction, proventriculus and gizzard junction, duodenum, Meckel's diverticulum, caecal tonsil, ileum and bursa of

Fabricsius. Among the organs, the bursa of Fabricius showed the most severe lesions including degeneration, necrosis, inflammation, haemorrhage, follicular lymphoid cells depletion, and follicular cyst formation. The virus induced degeneration, necrosis and depletion in the lymphoid cells of the GALT in the rest of the organs at days 2, 3 and 4 post inoculation (pi). The finding in this study showed that the acidic (pH 2.6) of proventriculus may hamper the infectivity or population of the virus. Thus, the following oral inoculation of vvIBDV, the virus is primary multiplied in the upper part of the GALT, at the junction between the oesophagus and proventriculus within 6 to 12 hours pi, rather than the lower GALT leading to primary viraemia.

it was concluded that all the Iranian IBVDV isolates were successfully isolated, identified and characterised as vvIBDV (except IR298 isolates) using conventional and molecular methods. They showed similar characteristic of vvIBDV reported from Europe, Japan and Hong Kong, except IR298 isolate showed the characteristics similar to the Chinese and Egyptian classical strains as well as vaccine strains. Inoculation of IR499 isolate (vvIBDV) in the SPF chickens produced the most severe lesions in the bursa of Fabricius at days 2, 3, 4 and 10 pi when compare to the GALT in the other organs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENCIRIAN MOLEKUL DAN PATOGENESITI VIRUS PENYAKIT BURSAL  
BERJANGKIT DIASINGKAN DI IRAN**

**Oleh**

**MOHAMMAD ALI BAHMANINEJAD**

**April 2004**

**Pengerusi: Profesor Madya Dr. Mohd Hair-Bejo, Ph.D.**

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Penyakit bursal berjangkit (IBD) adalah merupakan salah satu penyakit yang terpenting dalam industri ayam di Iran. Sepuluh virus IBD (IBDV) dari kes wabak di lapangan diperolehi. Isolat ini datang dari ayam pedaging dan penelur yang divaksin dan tidak divaksin dengan IBD di Iran dan dikenalpasti sebagai IR197, IR297, IR198, IR298, IR398, IR199, IR299, IR399, IR499 dan IR599.

Isolat ini menunjukkan tanda klinikal, kematian, lesi mata kasar dan histologi pada ayam komersil serupa dengan wabak IBD di lapangan. Isolat IBDV Iran ini diasingkan dan dikenalpasti melalui kaedah konvensional termasuklah AGPT, inokulasi telur, tisu didik CEF, transmisi mikroskop electron (TEM) dan pewarnaan immunoperoksidase sebagai IBDV. Isolate ini tidak membiak dan tidak menghasilkan CPE pada tisu didik CEF kecuali IR298.

IR298 dan IR499 telah diinokulasi ke atas ayam SPF berumur 28 hari; IR298 tidak menyebabkan kematian dan lesi mata kasar pada ayam SPF, walaubagaimanapun

atrofi berlaku ke atas bursa, manakala IR499 menyebabkan 70% kematian dan lesi matakasar dan histologi mempunyai lesi mirip vvIBDV.

Kesemua sepuluh isolat telah dicirikan melalui teknik molekul melalui RT-PCR-RFLP dan nested PCR. Kawasan hipervariabel gen VP2 dihasilkan dan jujukan serta pokok filogenetik dibentuk berdasarkan jujukan keseimbangan isolat Iran dan strain IBDV yang telah diterbitkan. Kesemua IBDV isolat mempunyai ciri vvBDV sama seperti laporan terdahulu, kecuali IR298 menunjukkan ciri strain vaksin. Analisis pokok filogenetik isolat ini menunjukkan ke semua isolate, kecuali IR 298 subkumpulan dengan vvIBDV serotip 1. Isolat IR197, IR299, IR399, IR398, IR499 dan IR599 menghasilkan cabang ketara dalam subkumpulan strain vvIBDV. IR298 berada dalam subkumpulan strain vaksin. Asal usul isolat ini mungkin sama seperti strain dari Eropah, Jepun dan Hong Kong manakala IR298 sama seperti strain klasikal China dan Mesir juga strain vaksin.

IR499 isolat ( $10^{6.7}$  EID<sub>50</sub>) telah diinokulat ke atas ayam SPF berumur 28 hari untuk mengenalpasti tindakbalas tisu limfoid di organ penghadaman (GALT) kepada isolat vvIBDV Iran. GALT adalah merupakan sekumpulan limfoid sel terdapat diantra esofagus dan proventriculus, persimpangan proventriculus dengan mepadal, duodenum, diverticulum Meckel's, tonsil sekum, ileum dan bursa Fabricius. Berbanding dengan tisu lain, organ bursa menunjukkan lesi yang paling teruk termasuklah degenerasi, nekrosis, inflamasi, perdarahan, pengurangan sel limfoid folikular dan pembentukan sist folikular.

Virus ini juga merangsang degenerasi, nekrosis dan pengurangan sel limfoid pada organ GALT lain pada hari ke 2, 3, dan 4 postinokulasi (pi).

Penemuan kajian ini menunjukkan proventrikulus yang asidik (pH 2.6) mungkin mengurangkan infektiviti atau populasi virus. Oleh itu, berikutan inokulasi vvIBDV secara oral, virus akan membiak terutamanya dibahagian atas GALT, pada persimpangan di antara oesopagus dan proventriculus dalam jangkamasa 6 hingga 12 jam p.i, berbanding dengan GALT bawah, dan seterusnya menyebabkan viremia primer.

Kesimpulannya, kesemua isolate IBDV Iran telah berjaya diasingkan, dikenalpasti dan dicirikan sebagai vvIBDV (kecuali isolat IR298) dengan menggunakan teknik konvensional dan molekul.

Kesemua isolat menunjukkan ciri yang sama seperti vvIBDV yang telah dilaporkan di Eropah, Jepun dan Hong Kong kecuali IR298 isolat menunjukkan ciri strain sama seperti China dan Mesir dan juga strain vaksin. Inokulasi IR499 vvIBDV isolat ke atas ayam SPF menghasilkan lesi yang paling teruk pada bursa jika dibandingkan lesi pada GALT di organ yang lain.

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I certify that an Examination Committee met on .....2004 to conduct the final examination of Mohammad Ali Bahmaninejad on his Doctor of Philosophy thesis entitled "Molecular Characterisation and Pathogenicity of Infectious Bursal Disease Virus Isolated in Iran" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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MOHAMMAD ALI BAHMANINEJAD

Date:

## TABLE OF CONTENTS

<b>DEDICATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	vi
<b>ACKNOWLEDGEMENTS</b>	ix
<b>APPROVAL</b>	xi
<b>DECLARATION</b>	xiii
<b>LIST OF TABLES</b>	xviii
<b>LIST OF FIGURES</b>	xix
<b>LIST OF ABBREVIATIONS</b>	xxi

### CHAPTER

<b>I</b>	<b>INTRODUCTION</b>	1
<b>II</b>	<b>LITERATURE REVIEW</b>	10
	Infectious Bursal Disease Virus	10
	IBDV Genome	12
	IBDV Proteins	14
	Resistance to Chemical and Physical Agents	15
	IBDV Replication	16
	Transmission	19
	Pathogenicity and Pathogenesis	19
	Clinical Signs	24
	Gross Lesions	25
	Histopathology	27
	Immunosuppression	29
	Antigenic and Virulence Variation	31
	Diagnosis	34
	Prevention and Control	39
	Phylogenetic Analysis	48
<b>III</b>	<b>ISOLATION AND IDENTIFICATION OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN IRAN</b>	
	Introduction	54
	Materials and Methods	58
	IBD Field Outbreaks	58
	History of Isolates	58
	Necropsy	61
	Histopathology	61
	Immunoperoxidase Staining	61
	Transmission Electron Microscopy (TEM)	62
	Samples Processing	63
	Agar Gel Precipitation Test (AGPT)	63
	IBDV Inoculation via Chorioallantoic Membrane (CAM)	64



IBDV Purification	65
Negative Staining	65
Enzyme Linked Immunosorbent Assay (ELISA)	66
Chicken Embryonic Fibroblast Cell Culture (CEF)	67
Specific Pathogen Free Chickens (SPF)	68
IR298IBDV isolate	68
IR499IBDV isolate	68
Results	69
Gross Lesions	69
Histopathology	72
Immunoperoxidase Staining	75
Agar Gel Precipitation Test (AGPT)	75
Enzyme Linked Immunosorbent Assay (ELISA)	76
Embryonated SPF Chicken Eggs via CAM Route	76
IBDV purification and Negative Staining	78
Transmission Electron Microscopy (TEM)	79
Specific Pathogen Free Chickens (SPF)	79
Control Group	79
IBD Groups	80
IR298IBDV isolate	80
IR499IBDV isolate	82
Chicken Embryonic Fibroblast Cell Culture (CEF)	82
Discussion	84
Conclusion	88

#### **IV MOLECULAR IDENTIFICATION AND CHARACTERISATION OF THE INFECTIOUS BURSAL DISEASE VIRUS ISOLATED IN IRAN**

Introduction	89
Materials and Methods	92
IBDV Isolates	92
RNA Extraction	92
Determination of RNA Concentration and Purity	93
Primers Design	93
cDNA Synthesis and PCR Amplification	94
Nested PCR Amplification	95
Agarose Gel Electrophoresis	95
Ethidium Bromide Staining	96
Purification of PCR Products	96
Determination of PCR Products Concentration	96
Sequencing of the PCR products	97
Cycle Sequencing	97
Purification of Cycle Sequencing Products	97
Denaturing Polyacrylamide Gel Electrophoresis	98
Restriction Fragment Length Polymorphism Analysis	99
Results	99
Quantification of RNA and PCR products	99
Amplification of Hypervariable Region of VP2 Gene	99
Nucleotide and Amino Acid Sequence of the Hypervariable Region	102



	Nucleotide Sequence of the Hypervariable Region	102
	Amino Acids Sequence of the Hypervariable Region	105
	Restriction Fragment Length Polymorphism Analysis	106
	Discussion	107
	Conclusion	111
<b>V</b>	<b>SEQUENCING AND PHYLOGENETIC ANALYSIS OF HYPERVARIABLE REGION OF VP2 GENE OF IBDV ISOLATED IN IRAN</b>	
	Introduction	112
	Materials and Methods	115
	Multiple Sequencing Assembly and Analysis	115
	Phylogenetic Tree Construction	117
	Results	117
	Nucleotides Sequence Analysis	117
	Percent Homology	130
	Deduced Amino Acids Analysis	130
	Phylogenetic Analysis	135
	Discussion	138
	Conclusion	141
<b>VI</b>	<b>RESPONSE OF THE GUT ASSOCIATED LYMPHOID TISSUES TO IRANIAN vvIBDV ISOLATE</b>	
	Introduction	143
	Materials and Methods	145
	IBDV Inoculum	145
	IBDV Titration	145
	Experimental SPF Chickens	146
	Bursa to Body Weight Ratio	147
	Histopathology	147
	Histological Lesion Scoring	147
	Immunoperoxidase Staining	149
	Statistical Analysis	149
	Results	150
	Clinical Signs in SPF Chickens	150
	Control Group	150
	IBD Group	150
	Gross Lesions	151
	Control Group	151
	IBD Group	151
	Histological Lesions	152
	Control Group	152
	IBD Group	152
	Bursa of Fabricius	152
	Caecal Tonsil	153
	Meckel's Diverticulum	154

Duodenum	154
Ileum	155
Oesophagus and Proventriculus	155
Proventriculus and Gizzard	156
Immunoperoxidase Staining	160
Control Group	160
IBD Group	161
Bursa of Fabricius	161
Caecal Tonsil	161
Meckel's Diverticulum	161
Duodenum	162
Oesophagus and Proventriculus Junction	162
Proventriculus and Gizzard Junction	162
Body Weight	165
Bursa Weight	165
Bursa to Body Weight Ratio ( $10^{-3}$ )	166
Discussion	170
Conclusion	173
<b>VII GENERAL DISCUSSION AND CONCLUSION</b>	<b>175</b>
<b>REFERENCES</b>	<b>183</b>
<b>APPENDICES</b>	<b>213</b>
A Chemicals and Reagents	213
B Fifty Percent Embryo Infective Dose per ml (EID <sub>50</sub> /ml) of the vvIBDV (IR499)	215
C Quantification of RNA Extraction of the Iranian IBDV Isolates	217
D Homology and Divergence Percentage of Nucleotide Sequence of the Iranian IBDV Isolates with the other IBDV Strains	218
E Lesion Scoring of the Organs	222
F Buffers and Media	223
G IBD Antibody Titer of IR399, IR499 and IR599 Isolates Obtained from the Field Outbreaks in the Non-Vaccinate Broiler Flocks	224
<b>BIODATA OF THE AUTHOR</b>	<b>225</b>

## LIST OF TABLES

Table		Page
3.1	History of IBD field outbreaks	60
4.1	Used primers for amplification of hypervariable region of VP2 gene of IBDV	94
4.2	Restriction enzyme site of nine Iranian Isolates	106
5.1	Characteristic of IBDV strains used for sequences comparison	116
5.2	Nucleotides differences of the Iranian isolates with published strains	133
5.3	Deduced amino acids differences of the Iranian isolates with published strains	134
6.1	Lesion scoring of the bursa of Fabricius.	148
6.2	Lesion scoring for the GALT at the junction between oesophagus and proventriculus, proventriculus and gizzard, Meckel's diverticulum, duodenum, ileum and caecal tonsil.	149
6.3	Comparison of the body weight, bursa weight and bursa to body weight ratio of the chickens between the control and IBD groups	169

## LIST OF FIGURES

Figure		Page
3.1	Haemorrhage in the thigh muscle of the chicken during IBD field outbreak, IR199 isolate	71
3.2	Severe haemorrhage in the bursa of Fabricius of the chicken during IBD field outbreak IR499 isolate	71
3.3	Haemorrhage at the junction of proventriculus and gizzard during IBD field outbreak, IR299 isolate	72
3.4	Follicular necrosis with cell debris, infiltration of inflammatory cells and enlargement of the interstitial space in the bursa of Fabricius of chicken during IBD field out break, IR198, X100, HE.	74
3.5	Positive reaction in the follicle of bursa of Fabricius of chickens during IBD field outbreak, IR398, x200, IPS	75
3.6	AGPT positive reactions between tested IBDV isolates and IBD antibody: (a) Positive control antigen, (b) Negative control (PBS) and (s) Tested IBDV isolates.	76
3.7	Generalized oedema and haemorrhage in the embryo of IBDV (IR499) infected embryonated SPF chicken eggs at day 3 pi.	78
3.8	Crystalline array IBD particles in the cytoplasm of lymphocytes in the bursa of Fabricius, IR499X30000,TEM.	79
3.9	Atrophied bursa of Fabricius after inoculation with IR298 in SPF chicken in comparison with the normal bursa of Fabricius at 7 days pi.	81
3.10	Follicular necrosis and atrophy, enlargement of interstitial connective tissue and corrugated epithelial lining cells in the bursa of Fabricius of SPF chicken inoculated with IR298 at 7days pi, x40, HE.	81
3.11	CPE in the CEF cell culture inoculated with IBDV isolate (IR298) at day 2 pi, X200.	83
4.1	RT-PCR product (643 bp) of the VP2 variable region of the Iranian IBDV isolates. Nucleotide position from 587 to 1229 based on Liu <i>et al.</i> , 1994.	100
4.2	Nested PCR product (552bp) of the VP2 variable region of the Iranian IBDV isolates. Nucleotide position from 651 to 1202 based on Kataria <i>et al.</i> , 1998.	101



4.3	Hypervariable region sequence of the Iranian IBDV isolates.	102
4.4	Amino acid sequence of the Iranian IBDV isolates.	105
5.1	Nucleotide sequence alignment of hypervariable region of VP2 gene of the Iranian IBDV isolates.	119
5.2	Deduced amino acid sequences of hypervariable region of VP2 of the Iranian IBDV isolates.	127
6.1	Lesion scoring of the organs in the IBD group throughout the trial	157
6.2	Severe haemorrhages and necrosis of the follicles with fibrinous exudates and cell debris in the medulla of the bursa of Fabricius of SPF chicken inoculated with vvIBDV isolate (IR499) at day 2 pi, HE, X100.	158
6.3	Follicular atrophy, degeneration, necrosis and vacuolization with infiltration of fibroblast and mononuclear inflammatory cells in the interstitial connective tissue in the bursa of Fabricius of SPF chicken inoculated with vvIBDV isolate (IR499) at day 10 pi, HE, X100.	158
6.4	Mild degeneration, necrosis and depletion of lymphoid cells in the GALT at the oesophagus and proventriculus junction of SPF chicken inoculated with vvIBDV isolate (IR499) at 6 hours pi, HE, X100.	159
6.5	Negative reaction in the bursa of Fabricius of SPF chicken in the control group at day 3 of the trial, IPS, X40.	160
6.6	Positive reaction (brown stain) mostly in the necrotic cells in the follicles of the bursa of Fabricius of SPF chicken inoculated with vvIBDV isolate (IR499) at day 3 pi, IPS, X200.	163
6.7	Positive reaction (brown stain) in the germinal centre of GALT in the Meckel's diverticulum of SPF chicken inoculated with vvIBDV isolate (IR499) at day 2 pi, IPS, X200.	163
6.8	Positive reaction (brown stain) in the germinal centre of GALT in the caecal tonsil of SPF chicken inoculated with vvIBDV isolate (IR499) at day 3 pi, IPS, X200.	164
6.9	Body weight of the chickens in the control and IBD groups	167
6.10	Bursa weight of the chickens in the control and IBD groups	167
6.11	Bursa to body weight ratio of the chickens in the control and the IBD groups	168



## LIST OF ABBREVIATION

ABTS	2,2-Azino-Bis (3-Ethylbenzthiazoline-6-Sulfonic acid)
AC-ELISA	Antigen captured-ELISA
AGPT	Agar gel precipitin test
ANOVA	Analysis of variance
APS	Ammonium persulfate
BF	Bursa of Fabricius
BGM	Baby grivet monkey kidney
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
cDNA	Complementary deoxyribonucleic acid
CEB	Chicken embryo bursa
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
CER	Chicken embryo rough
CMI	Cell mediated immunity
CPE	Cytopathic effect
CsCl	Caesium chloride
DAB	Diaminobenzadine HCl
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSA-ELISA	Double sandwich antibody-ELISA
dH <sub>2</sub> O	Distilled water
dsRNA	Double stranded RNA
DXV	Drosophilae X virus
EDTA	Ethylene diamine tetra acetic acid
EM	Electron microscopy
EID <sub>50</sub>	Embryo infective dose fifty
ELISA	Enzyme link immunosorbent assay
FCS	Foetal calf serum
FRET	Fluorescence resonance energy transfer
GALT	Gut associated lymphoid tissue
HRP	Horse radish peroxidase
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IBDV-BDA	IBDV-bursal disease antibody
IF	Immunofluorecent
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPNV	Infectious pancreatic necrosis virus
IPS	Immunoperoxidase staining
kDa	Kilo dalton
kV	Kilovolt
LE	lymphoepithelium
Mab	Monoclonal antibody

MDV	Marek's disease virus
MHC	Major histocompatibility complex
NCBI	National Centre for Biotechnology Information
NCR	Non coding region
NJ	Neighbour joining
nm	Nanometer
OD	Optical density
ORF	Open reading frame
OV	Oyster virus
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHYLIP	Phylogenetic interference package
pi	Post inoculation
pmol	Picamol
PTA	phosphotungstic acid
QAGPT	Quantitative agar gel precipitin test
RdRp	RNA dependent RNA polymerase
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
RT-PCR	Reverse transcriptase-PCR
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SPF	Specific pathogen free
SPSS	Statistical package for social science
STC	Standard challenge strain
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEM	Transmission electron microscopy
TEMED	N,N,N',N'-tetramethylenediamine
TNE	Tris-NaCl-EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3 propandiol
TV	Telina virus
UPGMA	Unweighted pair group with arithmetic mean
UV	Ultraviolet
VN	Virus neutralization
VNF	Virus neutralizing factor
VP	Viral protein
v/v	Volume per volume
vv	Very virulent
w/v	Weight per volume

## Amino Acids Code

Amino acid	3 letter designation	1 letter designation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid/ Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid/ Glutamate	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V