MOLECULAR CHARACTERIZATION OF CHICKEN INFECTIOUS ANAEMIA VIRUS AND ANTI-NEOPLASTIC EFFECT OF IT'S VP3 PROTEIN

IZATNAGAR

Thesis

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BY

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It is further certified that **Dr. N. Senthilkumar** has worked for about 30 months in this Institute and has put in more than 200 days attendance under me from the date of registration for the **Ph.D. degree** of this Deemed University, as required under the relevant ordinance.

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Dedicated to……

My parents and the voiceless birds who beared all the pains for me.

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ABBREVIATIONS

bp	:	Base pair
CIA	:	Chicken infectious anaemia
CAV	:	Chicken anaemia virus
CMI	:	Cell-mediated immune response
CO_2	:	Carbon dioxide
Con-A	:	Concanavalin-A
CPE	:	Cytopathic effects
^0C	:	Degree Celsius
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleoside triphosphates
DPI	:	Days post infection/immunization
EDTA	:	Ethylenediamine tetra-acetic acid
ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal bovine serum
FITC	:	Fluorescein isothiocyanate
GM	:	Growth medium
H&E	:	Haematoxylin and Eosin
HEPES	:	N-2-hydroxy ethyl piperazine-N'-2 ethane sulphonic acid
hr	:	Hour(s)
I/M	:	Intra-muscularly
IIFT	:	Indirect immunofluorescent technique
IU	:	International Unit
kb	:	Kilobase
kDa	:	Kilodaltons
LTT	:	Lymphocyte transformation test
M	:	Molar
MDCC-MSB1 cells	:	Marek's disease virus transformed chicken lymphoblastic T-
MDV	:	Marek's disease virus
µg	:	Microgram(s)
µl	:	Microlitre(s)
mg	:	Milligram(s)
$MgCl_2$	:	Magnesium chloride
min	:	Minute(s)

ml	:	Mililitre(s)
mm	:	Millimeter(s)
mM	:	Millimolar
MTT	:	3-(4,5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide
NaCl	:	Sodium chloride
ng	:	Nanogram(s)
OD	:	Optical density
ORF	:	Open reading frame
PBMCs	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PCV	:	Packed cell volume
pg	:	Picogram(s)
pH	:	-Log hydrogen ion concentration
pmol	:	Picomole(s)
RBCs	:	Red blood cells
RE	:	Restriction endonuclease enzyme
rpm	:	Revolutions per minute
RPMI-1640	:	Roseweli Park Memorial Institute-1640
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulphate
SI	:	Stimulation index
SPF	:	Specific pathogen free
ss	:	Single stranded
Taq	:	*Thermus aquaticus*
TBE	:	Tris-borate-EDTA
$TCID_{50}$	:	Fifty percent Tissue culture infective dose
TE	:	Tris-EDTA
TNE	:	Tris-NaCl-EDTA
Tris	:	Tris-hydroxy methyl aminoethane
UV	:	Ultraviolet
w/v	:	Weight/Volume

CONTENTS

INTRODUCTION

Chicken anaemia virus (CAV), belonging to *Gyrovirus* genus of *Circoviridae* family is the causative agent of chicken infectious anaemia (CIA), an immunosuppressive disease primarily of young chickens but also infects the chickens of all age groups. Chicken is the only recognized natural host, but serological survey has revealed the prevalence of this disease in domestic and wild birds (Farkas *et al.*, 1998). The clinical disease is mainly noticed in young chicks of 10 – 14 days of age, which usually acquire the infection vertically. The disease is characterized by increased mortality, reduced weight gain, anaemia, aplasia of bone marrow and atrophy of thymus (McNulty, 1991; Pope, 1991; Coombes and Crawford, 1996; Bulow and Schat, 1997; Rosenberger and Cloud, 1998, Dhama *et al.*, 2002). The major economic loss caused by this virus are associated with severe immunosuppression and increased mortality due to secondary bacterial/virus infections.

This virus, first reported by Yuasa *et al.* (1979) from contaminated vaccines used in Japan, has worldwide distribution. It is one of the smallest avian virus 23-25 nm in size, icosahedral, non-enveloped having a 2.3 kb circular single stranded DNA genome. The genome codes for three viral proteins (VP1, VP2 and VP3) transcribed from single major transcript of 2.0 kb size from three overlapping reading frames (ORF1, 2 & 3). Translation of the protein using internal start codons is the unique feature of this virus. The viral genome replicates through rolling circle model. Normally, the virus will not grow in any of the commonly used primary cells and cell lines. The Marek's disease virus or avian leucosis virus transformed lymphoblastoid cell lines are only susceptible to this virus and the virus usually multiply with low titre. Among the viral proteins, VP1 is the major capsid protein (54 kDa) and the only protein that could be detected in the SDS-PAGE analysis. VP2 is probably a non-structural protein found in the cells in early hours of virus replication cycle. VP1 and VP2 are the protective proteins inducing neutralizing antibodies (Koch *et al.*, 1995).

VP3 is the smallest protein of 13 kDa, called "apoptin" having unique apoptosis inducing property. It's unique nature of killing the neoplastic cells and not the normal cells by inducing apoptosis has made it a potential candidate to be used as an anti-neoplastic drug. P^{53} protein is the intracellular mediator of apoptosis through which almost all the anti-

cancer chemotherapeutic agents produce anti-cancer effect via the induction of this mediator and thereby causing the tumor cell to undergo natural cell death. Cancer cells develop resistance to the therapy by causing mutations in p^{53} gene and they escape from apoptosis. But, it had been proven that apoptin could induce apoptosis independently without p^{53} protein (Zhuang *et al.,* 1995). Peterson and Noteborn (2000) have reported that it could be used as a potential drug against the cancer cells resistant to current chemotherapeutic agents. However, the anti-neoplastic effect of this protein in virus-induced tumors has not been studied yet.

In India, Venugopalan and co-workers first reported the existence of CAV in 1994 and was later confirmed by Kataria *et al.* (1999). Dhama (2002) has characterized six isolates of CAV from different geographical regions of the country by RE analysis of PCR amplified 453 bp product and found variations among the isolates. Not much information is available about the chicken anaemia viruses (CAVs) circulating in this subcontinent. In general, no significant antigenic or pathogenic difference has been found among the reported isolates. Thus, only one serotype has been recognized so far. However, an antigenically different isolate (CAV-7) has been reported recently from USA (Spackman *et al.,* 2002), which could be a prototype virus of serotype 2. An outbreak in nine weeks old layer pullets with high morbidity (80%) and mortality (55%) in Taiwan has also been reported recently (Hsu JungPin *et al.*, 2002). These reports insisted the need of more emphasis to study and monitor the CAVs circulating in the field. CAVs isolated from many countries have been sequenced completely and phylogenetic analysis was made to find out the origin and relationship with other viruses. It has been reported that CAV isolates show extremely limited genetic variability ranging from 3-7% only. Renshaw *et al.* (1996) reported a hypervariable region (HVR) in VP1 protein spanning 13 amino acids (139-151). They also reported the amino acid at positions 139 and 144 are vital for the growth and spread of CAV isolates. It has also been documented that amino acid at position 394 in VP1 could be a major genetic determinant of virulence (Yamaguchi *et al.,* 2001). If it is glutamine at this position, the isolates are highly pathogenic and if it is histidine, less pathogenic. Hence, characterization of the Indian isolates at genome level is mandatory to know the exact nature of the virus prevalent in the country. Ultimately, it will help to formulate effective prevention and control measures in this subcontinent.

Control measures are mostly directed at limiting vertical transmission and subsequent clinical disease outbreaks in young chicks by vaccination of the parent flock before coming into lay. Acquired immunity efficiently prevents vertical transmission in the flock. The effective approaches followed include controlled exposures by artificially transferring the infected litter material, autogenous vaccine comprising of affected tissue (particularly liver) homogenates and a live wild-type virus vaccine administered in drinking water, but are risky due to residual virulent virus and/or contamination with other avian viruses (Vielitz and Landgraf, 1986, 1988; Vielitz *et al.,* 1987, 1989; McNulty, 1991). Live vaccine are prepared either using chicken embryo propagated or cell culture propagated CAV isolates, which had undergone multiple passages but harboured risks of reversion to virulence even after many passages (Vielitz *et al.,* 1987, 1989, 1991; Vielitz, 1989a,b; Rosenberger, 1992b). MSB1 cells are also productively infected with MDV (Todd *et al.,* 1995, 98). Therefore, conventional vaccines against CAV are not completely successful. Pages *et al.* (1997) developed an oil emulsified formalin inactivated CAV vaccine from MSB1 pool of CAV which proved to be highly immunogenic and protective against the disease in most of the progeny of the vaccinated breeders upto the required first 3-4 wks of life. Also, conventional approaches have difficulties due to the inability of the virus to grow to high titre in embryos/cell cultures (*in vitro*) and the unavailability of naturally occuring apathogenic isolates of CAV. Alternatively, development of recombinant vaccine using the immunogenic viral proteins will serve the purpose. Koch *et al.* (1995) developed an effective sub-unit vaccine using recombinant baculovirus insect cell system synthesizing and expressing CAV proteins VP1 and VP2, simultaneously and found these two proteins gave higher level of protection when administered together rather than given individually. Production of attenuated isolates by inducing mutations on the 12 nt sequence within the array of 19 nt repeats, associated with enhancer-promoter activity, have been reported (Notebom *et al.*1998b). Recently, Yamaguchi *et al.* (2001) highlighted that a single amino acid at position 394 of the VP1 as major determinant of pathogenicity and a point mutation at that position could generate a low pathogenic CAV which could become a potential candidate for developing live vaccine for CAV. Development of a naked DNA vaccine using the immunogenic protein genes of CAV (VP1 and VP2) could be an another useful approach for the prevention and control of this disease.

Keeping the above mentioned facts viz., molecular characterization of the genome of CAV isolates prevalent in India and the role of its proteins i.e. immunogenic role of VP1 and VP2 and the anti-neoplastic effect of the VP3 protein in view, the present study was envisaged with the following **objectives:**

1. **To detect variation, if any, among Indian CAV field isolates by RE analysis of PCR amplified VP1 and VP2 genes and sequencing of the viral genome to study their relationship with the reported CAV isolates from abroad.**
2. **To study the anti-neoplastic effect of CAV-VP3 protein in chicken experimentally infected with Rous sarcoma virus.**

REVIEW OF LITERATURE

Chicken infectious anaemia (CIA), an emerging disease mainly of young chicken, characterized by poor weight gain, severe anaemia, aplasia of the bone marrow, lymphoid atrophy, subcutaneous and muscular haemorrhages and increased mortality, has been responsible for considerable health problems and economic losses to the poultry industry (McNulty, 1991; Bulow and Schat, 1997; Hagood *et al.*, 2000). The causative agent, chicken anaemia virus (CAV), first reported by Yuasa *et al.* (1979), recently classified within the newly established genus *Gyrovirus* under the virus family *Circoviridae* (Pringle, 1999) is the smallest DNA virus and is now being recognized as an important avian pathogen worldwide (McNulty *et al.*, 1991; Farkas *et al.*, 1991; McIlroy *et al.*, 1992; Bulow and Schat, 1997; Rosenberger and Cloud, 1998). CAV has been proven to be a potent immunosuppressive agent for very young unprotected chicks thereby increasing their susceptibility to secondary infections viz. viral, bacterial and fungal agents and depressing vaccinal immunity and production performance in the field situations (Van Den Berg, 1996; Adair, 2000; De Herdt *et al.*, 2001). The virus seems to play a key role in the etiology of several multifactorial diseases viz. haemorrhagic syndrome, haemorrhagic anemia syndrome, infectious/aplastic anaemia, anaemia-dermatitis syndrome, gangrenous dermatitis and blue wing diseases (Bulow, 1991a; Pope, 1991; Jorgensen, 1991; Toro *et al.*, 2000; Hagood *et al.*, 2000). Certain notable characteristics such as vertical transmission, detection in SPF eggs, its highly contagious, hardy and ubiquitous nature along with the potential for inducing marked immunosuppression have attracted the global poultry production systems towards the CAV infection (Todd, 2000).

1. ETIOLOGY

1.1 Classification: Chicken anaemia virus (CAV), one of the smallest animal viruses, alongwith two other single stranded (ss) DNA animal viruses - porcine circovirus (PCV) and psittacine beak and feather disease virus (PBFDV) has been classified as a *Circovirus* (family *Circoviridae*) on the basis of morphology and circular genome characteristics (Studdert, 1993; Lukert *et al.*, 1995). As CAV differs from other circoviruses in size, morphology, buoyant density, sedimentation coefficient, antigenic determinants and gene arrangement (Todd *et al.*, 1991; Noteborn and Koch, 1995; Bassami *et al.*, 1998), it has now been placed under a new genus named *Gyrovirus* under the family Circoviridae (Pringle, 1999; Todd *et al.*, 2000).

However, CAV has a genome organization similar to a recently described TT virus, the first human circovirus (Mushahwar *et al.,* 1999; Leary *et al.,* 1999). Therefore, CAV and unclassified TT virus might be the member of the same family, distinct from circoviruses (Santeen *et al.,* 2001). An antigenically different isolate (CAV-7) has been reported recently from USA (Spackman *et al.,* 2002), which could be a prototype virus of serotype 2.

1.2 Strain Differentiation: No significant antigenic differences have been recognised among various CAV strains using polyclonal antibodies. There exist only one serotype of CAV worldwide (Yuasa and Imai, 1986; McNulty *et al.,* 1990a; Bulow and Schat, 1997; Adair, 2000). However, monoclonal antibodies (MAbs) revealed antigenic differences, based on immunofluorescence reaction patterns on staining between isolates which are indistinguishable using polyclonal antisera (McNulty *et al.*, 1990c).

1.3 Physical and Morphological Characteristics: CAV has been characterized as a very small, naked, icosahedral DNA virus with spherical or hexagonal shape measuring 19.1 to 26.5 nm in diameter (avg. 23.5 to 25 nm) (Gelderblom *et al.,* 1989; McNulty *et al.,* 1990a,d; Todd *et al.,* 1994). Virions have been demonstrated to possess a buoyant density of 1.33-1.34 or 1.35-1.37 g/ml in cesium chloride (CsCl), sedimentation coefficient of 91S in isokinetic sucrose gradient and a circular covalently closed ss-DNA genome of 2.3 kb enclosed in a capsid of 32 capsomeres (Todd *et al.,* 1990a, 1991b; Allan *et al.,* 1994; Bulow and Schat, 1997).

1.4 Resistance to Physical and Chemical Agents: The structure of CAV confers remarkable chemical and thermal stability. Virus has the ability to resist inactivation by various physico-chemical agents viz. exposure to pH 3, lipid solvents (ether or chloroform), acetone and treatment for two hours at 37°C with 5% solutions of many commercial disinfectants (Yuasa, 1992; Taylor, 1992; Allan *et al.,* 1994; Bulow and Schat, 1997). Treatments with 1% glutaradehyde for 10 min at room temperature, 0.4% beta-propiolactone for 24 hr at 4^0C and 5% formaldehyde for 24 hr at room temperature have been recommended for complete inactivation of the virus (Yuasa, 1992). Iodine and sodium hypochlorite at 1% concentration can also inactivate the virus. The virus has been shown to remarkably withstand temperatures of 56°C or 70°C for 1 hr and upto 80°C for 15 min. Heating at a temperature of 100°C completely inactivated the virus within 15 min (Urlings *et al.,* 1993).

2. MOLECULAR CHARACTERISTICS

2.1 genome: The gene structure of the virus has been well characterized and the molecular biology of CAV has been studied by several workers (Noteborn *et al.,* 1991, 1994b; Classens *et al.,* 1991; Noteborn and Koch, 1995; Brown *et al.,* 2000; Santeen *et al.,*

2001). Analysis of the CAV genome revealed a circular, covalently linked negative sense ss-DNA genome of 2.3 kb containing only one promoter region and poly (A) addition signal. Thus far, all sequenced strains comprise of three partially overlapping major open reading frames (ORFs) -ORF-1/C1 (853-2199 nt position, 1347 nt), ORF-2/C2 (380-1027 nt position, 648 nt) and ORF-3/C3 (486-848 nt position, 363 nt), that are frame shifted encoding putative viral proteins VP1, VP2 and VP3, respectively (Todd *et al.,* 1990a; Chandratilleke *et al.,* 1991; Douglas *et al.,* 1995; Coombes and Crawford, 1996). The Japanese strain CAA 82-2 contains a fourth open reading frame (ORF-4), but its function has yet to be established (Kato *et al.,* 1995). The replicative form of the viral genome has been shown to consist of 2298 or 2319 bp depending on the presence or absence of four or five 21-bp repeats (Soine *et al.,* 1994; Todd *et al.,* 1995, 1996). A hypervariable (HV) region has been identified in the largest ORF/C1 spanning amino acid (AA) positions 139-151 (13 AA region) in the VP1 (Renshaw *et al.,* 1996).

2.2 Viral Proteins (VP) and Antigens: Three putative viral proteins viz. VP1 (45-52 kDa, 449 AA), VP2 (24-30 kDa, 216 AA) and VP3 (13.6-16 kDa, 121 AA) have been well characterized by several workers (Noteborn *et al.,* 1991; Classens *et al.,* 1991; Chandratilleke *et al.,* 1991; Meehan *et al.,* 1992; Buchholz, 1994; Coombes and Crawford, 1994). VP1 is the major structural capsid protein (Todd *et al.,* 1990a, 1994; Koch *et al.,* 1994), though VP2 detection in purified virions revealed it might also to be a structural protein (Buchholz and Bulow, 1994). VP3 is not present in purified CAV particles but is important for its life cycle (Noteborn *et al.,* 1994a,b). Both VP1 and VP2 are involved with antigenicity of the virus, and formation of neutralizing antibodies in CAV-infected cells require the synthesis of both VP1 and VP2 in the same cell (Koch *et al.,* 1994, 1995; Noteborn *et al.,* 1998a). VP3 is poorly immunogenic (Cunningham *et al.,* 2001). VP3 is the major protein found in infected cells produced in abundance during virus replication. There is a strong binding between VPs and cellular proteins as all are present in nuclear extracts/intranuclear inclusion (Todd *et al.,* 1990a, 1994; Douglas *et al.,* 1995). VP3 (apoptin) induces apoptosis in specific lymphoid cells, the chicken thymocytes and chicken lymphoblastoid cell lines (MSB1) (Jeurissen *et al.,* 1992b) which is also an important phenomenon during the pathogenesis of CAV (Noteborn *et al.,* 1994a; Noteborn and Koch, 1995; Chiu *et al.,* 2001). It can also induce p53-independent apoptosis in human osteosarcoma cells (Zhuang *et al.,* 1995).

2.3 Genomic Variations: CAV isolates show extremely limited genetic variability worldwide and the amino acid composition has been found to be remakably conserved. A low level (~3-7 %) of sequence diversity is detected when the isolates are compared, thus

reflecting high sequence identity among isolates and which is consistent with the finding that there exists only one serotype (Todd *et al.*, 1992, 1998; Pallister *et al.*, 1994; Kato *et al.*, 1995; Farkas *et al.*, 1996; Brown *et al.*, 2000; Santeen *et al.*, 2001). Renshaw *et al.* (1996) highlighted a possible functional role for the natural changes in the hypervariable (HV) region contributing to differences in efficiency of viral replication or rate of spread *in vitro*. CAV mutates during passage in cell cultures and the high passage virus is genetically and biologically diverse in pathogenicity and antigenicity (Meehan *et al.*, 1997; Scott *et al.*, 1999).

3. LABORATORY HOST

CAV can be propagated and assayed in susceptible chickens, in cell cultures or in chicken embryos. Day-old chicks, free of maternal CAV-antibodies, inoculated with CAV develop pronounced clinical disease at 12-16 days (Yuasa *et al.*, 1979, McNulty, 1991). The virus multiplies in the chicken embryos following yolk sac inoculation but does not reach a titre high enough to cause embryo death or produce noticeable gross lesions. However, the hatched chicks develop characteristic disease signs (Bulow and Witt, 1986; Tantaswasdi *et al.*, 1996; Brown *et al.*, 2000). CAV does not grow in routine standard cell cultures, but grows in some lymphoblastoid cell lines established from Marek's disease virus (MDV) and lymphoid leukosis lymphomas. The most commonly used of these is the continuous MDCC-MSB1 cell line derived from a MD T-cell splenic lymphoma (Yuasa, 1983; Adair *et al.*, 1993b; Coombs and Crawford, 1998). Also the sublines of MSB1 differ in their susceptibility to infection and even some CAV strains don't replicate in these cells (Renshaw *et al.*,1996; Calnek *et al.*, 2000).

Infected cell cultures show a general cytopathic effect. Cell-associated infectivity peaks at 36-42 hr after inoculation whereas cell-free titres peak at 48-72 hr after inoculation and the rate of multiplication ranges between 10-100 fold (Yuasa, 1983, Bulow *et al.*, 1985). Other cell lines suitable for propagation and assay include MDCC-JP2 (T cell, MDV transformed), LSCC-1104/X5 B1 (B-cell, induced by ALV), LSCC-HD11 (AMV transformed) and MDCC-CU147 (MD local lesion derived T-cell line) lymphoblastoid cell line (Jeurissen *et al.*, 1992b; Noteborn *et al.*, 1994a; Calnek *et al.*, 2000). Mononuclear cell cultures derived from various chicken lymphoid tissues supported CAV replication but with low virus titre yield as compared to infection in MSB1 cultures (McNeilly *et al.*, 1994). Although MSB1 cells are preferred, the significance of CU147 as an alternate cell line has also been realized (Calnek *et al.*, 2000).

4. VIRUS REPLICATION

By conventional adsorption and penetration virus probably enters the target cell and multiplies in the nucleus by circular ds-DNA replicative intermediate form (rolling circle model) (Meehan *et al.*, 1992; Noteborn *et al.*, 1992a, Phenix *et al.*, 1994). In chickens, CAV appears to replicate primarily in hematopoietic precursor cells in the bone marrow and thymic precursors (lymphocytes) in the thymus cortex where it leads to cytolytic infection and cell death by apoptosis (Jeurissen *et al.*, 1992b, Noteborn *et al.*, 1994a, Noteborn and Koch, 1995).

5. PATHOGENICITY / VIRULENCE

Non-pathogenic CAV isolate does not exist naturally. Isolates of CAV identified so far do not differ significantly in pathogenicity (Yuasa and Imai, 1986; Takagi *et al.*, 1996), however, lesions severity has been found to vary considerably in few pathogenicity studies (Goryo *et al.*, 1985, 1987a, 1989a; Lucio *et al.*, 1990; Lamichhane *et al.*, 1991; Toro *et al.*, 1997). Differences in ability to cause anaemia in chicks infected at 1 day of age have not been noted but can occur in these chicks inoculated at 5-8 days of age (Yuasa and Imai, 1986; McNulty *et al.*, 1990a). The ability of CAV to produce anaemia is directly related to the dose of virus inoculated and the age of susceptible host (Yuasa and Imai, 1986; McNulty *et al.*, 1990b) and its pathogenicity is enhanced by concurrent infection with other immunosuppressive agents (Coombes and Crawford, 1996). So the reported variability in virulence among isolates should not be interpreted as a proof of variation in pathogenicity. The genetic determinants of pathogenicity are not known except that VP3 causes death of infected cells via apoptosis (Noteborn and Koch, 1995). Recently it has been reported that one amino acid, at residue 394 of the VP1 capsid protein, serves as a major determinant of pathogenicity (Yamaguchi *et al.*, 2001). A pathogenically different isolate (CAV-7) has been reported recently from USA (Spackman *et al.*, 2002), which could be a prototype virus of serotype 2. An outbreak in nine weeks old layer pullets with high morbidity (80%) and mortality (55%) in Taiwan has also been reported recently (Hsu JungPin *et al.*, 2002).

6. ATTENUATION

Although it has been claimed that CAV can be attenuated progressively by extensive passages in cell cultures, but this has not been confirmed as the irreversible attenuation proved to be difficult because of the relatively simple genomic nature of the virus (Bulow and Fuchs, 1986a,b; Goryo *et al.*, 1987b; Bulow *et al.*,1991; Todd *et al.*,1995, 1998). Nucleotide changes in each of the three genomic regions contribute towards attenuation (Meehan *et al.*, 1997). CAV strains with a mutated enhancer/promoter region sharing reduced virus spread and cytopathogenicity have been reported (Noteborn *et al.*,

1998b), but whether attenuation is achieved is to be tested. Reversion to virulence has been reported even after 173 passages in MSB1 cells (Bulow and Fuchs, 1986b; Todd *et al.*, 1995). It is more likely that very highly passaged (>300 passages) cloned CAV isolates, possessing reduced pathogenicities as revealed by selection of 6 AA changes in VP1 coding regions, may be suitable candidates for live attenuated CAV vaccine development (Scott *et al.*, 1999; Todd *et al.*, 2001). A change at the residue 394 of VP1, the major determinant of pathogenicity of CAV, could help generate a low pathogenic CAV suitable for stable live vaccine development (Yamaguchi *et al.*, 2001).

7. THE DISEASE

CIA is a highly contagious disease with a high prevalence in countries with intensive poultry production. Clinical disease is very much uncommon since most breeders seroconvert to CAV either as a result of natural infection or vaccination of breeders before they come into lay so that the progeny of infected breeder stocks are protected by maternal antibodies. Much more common are subclinical infections in chicks above 3 weeks of age. Outbreaks of CAV are therefore sporadic in the field (Imai and Yuasa, 1990; Bulow and Schat, 1997; Todd, 2000).

7.1 Transmission: Vertical as well as horizontal mode of transmission are involved in the spread of CAV among chickens resulting in clinical and sub-clinical infections, respectively (Yuasa *et al.*, 1979, 1983b; Yuasa and Yoshida, 1983; McNulty, 1991; Hoop, 1992). Embryo infection after insemination with CAV-contaminated semen can also occur (Hoop, 1993). Vertical transmission occurs when breeder layers with no antibody to CAV or with no previous exposure to the virus become infected as they come into egg production. Clinical disease does not occur in breeders and there is no apparent effect on egg production, hatchability or fertility but the virus is passed vertically to the offspring which in turn develop clinical disease (Vielitz and Landgraf, 1988; Hoop, 1992). Vertical transmission occurred for 8-14 days after experimental infection of hens (Yuasa and Yoshida, 1983), but field observations indicated that egg transmission could occur during a period of 3-9 wk post-exposure with a peak at 1-3 wk (Engstrom and Luthman, 1984; Vielitz and Landgraf, 1988; Chettle *et al.*, 1989; Hoop, 1992). After the appearance of CAV-antibody, the excretion of CAV via the faeces ceases. So the infection is short lasting with elimination of CAV within 4-6 weeks PI supporting no evidence of latency (Yuasa *et al.*, 1983b; Hoop, 1992). However, Cardona *et al.* (2000b) recently reported wide distribution and long persistence of CAV in the reproductive tissues of infected SPF flocks of chickens indicating CAV can thus be vertically transmitted from persistently infected hens.

Horizontally acquired infection, usually occur in chickens lacking maternal antibody to CAV. Infection usually takes place by direct or indirect contact from virus surviving in poultry houses between flocks, from virus excreted by a small number of vertically infected hatchmates, or through ingestion of material contaminated with infected faeces (McNulty *et al.,* 1988, 1991; Bulow, 1988; Rosenberger and Cloud, 1989a,b; Jorgensen, 1990). Management practices may also have an important role in the outcome of horizontal CAV infections (Jorgensen *et al.,* 1994, 1995b).

Another possible method of spread is through contaminated vaccines because the virus has been detected in some SPF flocks used for vaccine production, raising the possibility that such vaccines might have been responsible for disseminating CAV infection in the past (Cardona *et al.,* 2000a). Experimentally, CAV can be transmitted by inoculation of the virus material in the susceptible day-old chickens by I/M or I/P routes (Yuasa *et al.,* 1979; McNulty, 1991; Brentano *et al.,* 1991; Kataria *et al.,* 1999; Dhama, 2002). Vertical transmission, being the major cause of clinical disease has more importance than horizontal spread (Chettle *et al.,* 1989; Hoop, 1992). CAV has no known public health significance.

7.2 Susceptibility: Chickens of all ages are susceptible to CAV infection but after two weeks of age, susceptibility to clinical disease decreases rapidly which is related to an age associated resistance primarily due to the development of immune competence to produce effective humoral response (Yuasa *et al.,* 1980a; Otaki *et al.,* 1992; Hu, 1992; Jeurissen *et al.,* 1992a; Hu *et al.,* 1993a; McConnell *et al.,* 1993a,b). For chicks less than 2-3 weeks of age, maternal antibodies are very protective for clinical disease, most breeder flocks then seroconvert as a result of horizontal infection generating protective humoral immunity. Both sexes are affected, broilers are found to be more susceptible with chicks at one day of age being most susceptible. (Yuasa *et al.,* 1979, 1980a; Vielitz and Landgraf, 1988, Rosenberger and Cloud, 1989b; Otaki *et al.,* 1992).

Chicks are at increased risk of infection with CAV and the period of susceptibility to disease may be extended by an early exposure to other lymphocidal agents such as IBDV, MDV, REV, adenovirus or selected avian reoviruses and other agents that interfere with normal immune system development (Engstrom *et al.,* 1988; Rosenberger and Cloud, 1989b; Bulow and Schat, 1997; Imai *et al.,* 1999) or by bursectomy (Yuasa *et al.,* 1988; Lucio *et al.,* 1990; Hu *et al.,* 1993a). A connection between the genetic strain of chicken and susceptibility to CAV infection has also been reported (Cardona *et al.,* 2000a). Production or transfer stress might enhance susceptibility to CAV infection or consequently persistent CAV infection may be activated because of hormonal changes leading to

vertical transmission and subsequent seroconversion near sexual maturity (McNulty *et al.*, 1991; Cardona *et al.*, 2000a,b).

7.3 Clinical Disease: Specific clinical disease is observed during the first 3-4 wk of life when chicks without maternally derived antibodies are infected vertically or horizontally in the first two weeks of life (Mcnulty, 1991; Bulow and Schat, 1997). The subclinical phase lasts until day 8-10 PI to be followed by a mild to severe anaemic phase between days 10 and 20 PI and then a convalescent phase from day 20 PI.

7.3.1 Signs: The disease is acute with clinical stage developing after an incubation period of 10-14 days, include inconsistent pathognomic symptoms of depression, paleness (anaemia), weakness, anorexia, ruffled feathers and stunted growth (particularly between 10-20 DPI) depending on disease severity. Anaemia, the only specific sign with a peak at 14-16 DPI is noticeable on the non-feathered areas such as the comb and wattles, eyelids and legs, and finally the bird become markedly pallor and that may extend to the internal organs (Yuasa *et al.*, 1979; Goryo *et al.*, 1985; Pope, 1991). It may, however, be more than 14-21 days before anaemia develops, presumably depending on genetic strain and age of experimental chicks, and properties of the inoculated virus strains (Rosenberger and Cloud, 1989b).

7.3.2 Morbidity and Mortality: Growth is retarded and mortality peaks within the third week of life and varies usually between 5% and 10% but upto 60% has been recorded (Engstrom and Luthman, 1984; Taniguchi *et al.*, 1982, 1983; McNulty *et al.*, 1991). Morbidity similarly varies between 20-60%. Usually no more than 30% of birds die between 12-28 DPI with peak mortality within 5-6 days of onset of disease (14-18 DPI). Morbidity and mortality may be influenced by the virulence, dose and route of the CAV strain (Goryo *et al.*, 1985, Yuasa and Imai, 1986). Under field conditions, congenitally infected chicks show clinical signs and increased mortality begining at 10-12 days of age, with a peak at 17-24 days. In heavily infected flocks, there can be a second peak of mortality at 30-34 days possibly due to horizontal infection (Goryo *et al.*, 1987a; Chettle *et al.*, 1989; Jorgensen, 1991).

7.3.3 Gross Lesions: Gross lesions in lymphoid tissues and bone marrow are most pronounced at 12-16 DPI. Lesions consist of transient severe bone marrow aplasia and pancytopaenia with reduction in hematocrit values ranging between 6-27% (PCV < 25%) due to severe anaemia. There is normochromic anaemia in CIA. The blood becomes watery and the plasma is paler, clotting time is increased (Taniguchi *et al.*, 1982, 1983; Goodwin *et al.*, 1991a, 1992b; Zhou and Liu, 1996; Liu *et al.*, 1997b; Ramadan *et al.*, 1998). Bone marrow characteristically changes from a red to a pale yellow to whitish colour

and might have a fatty consistency. The femur is most commonly evaluated for this change. Liver becomes pale, discoloured and enlarged. There is intense atrophy of the lymphoid organs at the peak of anaemic phase, including the thymus, bursa of Fabricius, and to a lesser extent spleen. A yellow fatty bone marrow is the most characteristic lesion due to anaemia, while thymic atrophy is the most consistent finding. Other visceral organs might show areas of congestion and hemorrhages. Bursal atrophy is generally modest and transitory (Yuasa *et al.,* 1979; Goryo *et al.,* 1985, 1987a, 1989a; Yuasa, 1989; Bulow and Schat, 1997). Lesions in other tissues including swollen and a mottled appearance with focal necrosis of the liver, kidney and spleen, erosions in the gizzard, skin necrosis on the wings, and consolidation of lungs have also been reported but are likely to be associated with secondary infection with other agents in the natural field disease. Also in field vertical CAV infection results in a more severe disease than chicks infected experimentally at day-old age (Bulow *et al.,* 1986b,c; Goryo *et al.,* 1987a; McNulty, 1991; Bulow and Schat, 1997). Sometimes haemorrhages occur throughout the body. Haemorrhages in the proventricular mucosa and subcutaneous and muscular haemorrhages within the wing tips are sometimes associated with severe anaemia. (Goryo *et al.,* 1985; Yuasa and Imai, 1986; Engstrom *et al.,* 1988).

7.3.4 Histopathology: Microscopic lesions reflect the macroscopic observations. The histologic changes do not always correspond with the clinical signs and haematocrit values. Panmyelopathy and transient generalized lymphoid atrophy (panleukopenia) are observed at the peak of the disease (Taniguchi *et al.,* 1982, 1983; Goryo *et al.,* 1989a; Chettle *et al.,* 1989; Pope, 1991). In the bone marrow there is atrophy and hypoplasia/aplasia involving all compartments and haematopoietic lineages. Both erythropoiesis and haematopoiesis are affected with mild to severe depletion of both erythroid and myeloid cells. Haematopoietic cells comprising erythrocytes, white blood cells (heterophils, monocytes and eosinophils) and thrombocytes are markedly reduced in numbers from respective intra- and extra-vascular spaces, leading to pancytopaenia or are replaced almost completely by lipocytes or proliferating stroma cells, giving the bone marrow a watery texture and the characteristic yellow colour (Taniguchi *et al.,* 1982, 1983; Lucio *et al.,* 1990; Liu *et al.,* 1997b). Large thymic lymphocytes are swollen 2-3 times in size. There is a well documented necrosis of the lymphoid elements in thymus, bursa, spleen, caecal tonsils and other tissues with the depletion of lymphocytes followed by reticular cell hyperplasia and connective tissue proliferation (Goryo *et al.,* 1989a; McNulty, 1991).

Histologically, lesions appear first in bone marrow and thymus on 6 DPI and then in the bursa, spleen and liver (Goryo *et al.,* 1989a,b,c; Smyth *et al.,* 1993). The thymus cortex and medulla become equally atrophic, with hydropic degeneration of residual cells and occasional necrotic foci. Lesions in BF consist of atrophy of the lymphoid follicles with occasional small necrotic foci, infolded epithelium, hydropic epithelial degeneration and proliferation of reticular cells. In the spleen, atrophy of lymphoid tissue with hyperplasia of reticular cells is seen in the lymphoid follicles as well as in the Schweigger-Seidl sheaths. In the liver, kidneys, proventriculus, duodenum, and caecal tonsils, lymphoid foci are depleted of cells, making them smaller and less dense than those in unaffected birds. Liver cells are swollen, hepatic sinusoids may be dilated and the lymphoid foci are depleted of cells. Focal necrosis and vacuolar degeneration in the liver, as well as apoptosis in different organs is evident.

Small eosinophilic intranuclear inclusion bodies (INIBs) have been detected in altered cells, mainly in large haematopoietic precursor cells in the bone marrow, and in swollen thymocytes (lymphoblasts) and hyperplastic reticular cells in the thymic cortex, in experimental infections most frequently at 6-8 DPI. Inclusions can also be seen in the spleen, liver, bursa and lymphoid aggregates throughout the body from 6 to 16 DPI. Inclusions can be considered pathognomic but being transient and small sized, identification is difficult, therefore, are of limited diagnostic value (Goryo *et al.,* 1989a; Pope, 1991; Smyth *et al.,* 1993; Toro *et al.*, 1997).

7.4. Factors affecting the disease: Uncomplicated infectious anaemia, especially if caused by horizontal infection, result in slightly increased mortality and transient poor performance of affected flocks, and therefore, it could even go unobserved in commercial settings. Severity or intensity of the disease is related to the viral dose, age of infection, the maternal antibody status and the route of infection (vertical/horizontal) (Yuasa *et al.,* 1980a,b; Rosenberger and Cloud, 1989b; McNulty *et al.,* 1990b). Oral, nasal, or ocular routes are much less effective than parenteral inoculation in inducing disease. Bursal atrophy is an important risk factor for the development of CIA (Yuasa *et al.,* 1988; Hu *et al.,* 1993a; Hagood *et al.,* 2000). Chemical immunosuppression by betamethasone or cyclosporin A also aggravates signs and lesions (Bulow *et al.,* 1987). All of the aforesaid signs and lesions may be exacerbated and become more persistent in chickens co-infected with other lymphocidal agents leading to retarded recovery and increased mortality which is frequently seen in field cases (Bulow *et al.,* 1983; Engstrom *et al.,* 1988; McNulty, 1991; Todd, 2000; Miles *et al.,* 2001).

7.5 Disease Status in India

In India, the disease has been suspected since long on the basis of clinical symptoms and lesions in the major poultry raising states of the country (Verma *et al.,* 1981; Suresh *et al.,* 1995). While investigating an outbreak of multifactorial etiology in young growing chickens in poultry belt of Namakkal, Tamil Nadu, Venugopalan *et al.* (1994) reported the disease by demonstrating CAV through immunoperoxidase test. Recently, Kataria *et al.* (1999) reported the existence of CIA from three states (Tamil Nadu, Maharashtra and U.P.) in India by PCR detection of CAV-DNA in the clinical samples and transmission study in chicks. Subsequently virus detection and isolations from other states further substantiated the occurrence of CIA in India (Dhama, 2002). Thus CAV may be placed in the list of emerging viruses which can cause severe threat to the Indian poultry industry (Goudar and Arun, 1992; Singh *et al.,* 1996; Kataria *et al.,* 2001, 2002a,b; Senthilkumar *et al.,* 2002). This warrants the need for determining the epidemiological status of the disease in the country, emphasizing CAV research for diagnosis and developing suitable control measures.

8. PATHOGENESIS

Chronological studies of the affected tissues revealed the pathogenesis of CAV infection (Taniguchi *et al.,* 1983; Goryo *et al.,* 1989a,b,c; Hoop and Reece, 1991; Smyth *et al.,* 1993) indicating primary target cells to be haematopoietic precursor (haemocytoblasts) and thymic precursor (lymphoblasts) cells in the bone marrow and thymus cortex, respectively, in early cytolytic infection at 6-8 DPI and also in reticular cells. CAV antigen has been detected in cells in bone marrow, thymus and spleen 3-4 DPI and subsequently in other tissues viz. liver, proventriculus, duodenum, lung, kidneys and heart, indicating its wide distribution throughout the body. No antigen has been detected after 26 DPI (Hoop and Reece, 1991; Adair *et al.,* 1993b; Smyth *et al.,* 1993) although virus might persist in tissues until 28 days and in rectal contents until 49 days or later (Yuasa *et al.,* 1983b). CAV replicates in lymphocytes, causing destruction of thymic lymphocytes, and is directly cytotoxic for bone marrow haematopoietic precursors leading to transient severe anaemia and immunosuppression (Taniguchi *et al.,* 1983; Goryo *et al.,* 1989a,b,c; Zhou and Liu, 1996; Liu *et al.,* 1997b; Adair, 2000). CAV has specific tropism for lymphoid tissues particularly for the thymus cortex (T-cells preferably), affecting lymphopoiesis so that there is depletion of thymic lymphblastoid cells and lymphocytes in the thymus (precursor T-cells), spleen (mature T-cells) and other lymphoid tissues (Goryo *et al.,* 1989a; Smyth *et al.,* 1993; Adair *et al.,* 1993b; McNeilly *et al.,* 1993). Thymic lymphocyte loss is more

severe than bursal lymphocyte loss (Jeurissen *et al.,* 1989; Hu *et al.,* 1993a,b; McNeilly *et al.,* 1994).

The thymocyte infection with CAV causes chromatin aggregation, fragmentation of cellular DNA into oligonucleosomes, karyorrhexis and cell death via apoptosis, and by day 7-14 PI, there is complete depletion of the thymic cortex. Nuclear inclusions are exclusively VP3-induced apoptic bodies (Jeureissen *et al.,* 1992b; Noteborn *et al.,* 1994a; Chiu *et al.,* 2001). Apoptosis represents an important phenomenon during the pathogenesis of CAV in which virus interferes with programmed cell death (Noteborn and Koch, 1995; Noteborn, 2001). Eosinophilic intranuclear inclusions have been found in altered cells especially in the bone marrow as well as in thymus (Goryo *et al.,* 1989a,b,c; Smyth *et al.,* 1993). Haemorrhages associated with CAV infection relates primarily to destruction of the thrombocytes causing thrombocytopaenia and impaired clotting (Cheville, 1983; Pope, 1991; Liu *et al.,* 1997b). Depletion of granulopoietic tissue is responsible for the lack of mounting inflammatory responses in the immunosuppressed chicks.

Repopulation of the bone marrow with proerythroblasts and promyelocytes, and recovery of haematopoietic activity (erythropoiesis) and lymphocyte repopulation after 16-20 days of inoculation appear to coincide with the beginning of antibody formation. These events result in complete recovery by 32-42 days (Taniguchi *et al.,* 1982, 1983; Goryo *et al.,* 1985; Liu *et al.,* 1997b). Pathogenesis of CAV infection is strongly related to humoral immunity (Yuasa *et al.,* 1983b, 1988).

9. IMMUNITY TO CAV INFECTIONS

9.1 Active Immunity: CIA can be effectively prevented by a humoral immune response in the immunocompetent host or with passively acquired maternal antibodies (Yuasa *et al.,* 1980a, 1983b, 85; Hu *et al.,* 1993a). Humoral (neutralizing) antibodies play a crucial role in age related resistance. Resistance to experimental reproduction of clinical disease is acquired rapidly after hatching which is normally complete by 2 weeks of age (Yuasa and Imai, 1986) and in older birds, is attributed to disappearance of susceptible cells (Jeurissen *et al.,* 1992a) or more likely to increased immunocompetence (Yuasa *et al.,* 1988; Hu, 1992; Hu *et al.,* 1993a). CAV antibodies persist for long time (Imai *et al.,* 1993) and only very low levels are needed to give effective protection (Otaki *et al.,* 1992). Vertical transmission of the virus is unlikely to occur from actively immune hens (Hoop, 1992).

Poor antibody response is shown by susceptible chickens inoculated with CAV at 1 day of age. CAV suppresses both general and local humoral immunity in chicks by decreasing the antibody producing cells in most of the immune organs at 7-28 days after infection of the birds at hatch (Liu *et al.,* 1997a; Zheng *et al.,* 1997). Neutralizing antibodies

are detected at 3 wk PI with low titres (1:80-1:320) that increases subsequently peaking at 4-5 wk from 1:1280 to 1:5120. Antibody response is considerably enhanced and faster in older chickens inoculated I/M at 2-6 wk of age with neutralizing antibody detectable as early as 4-7 days. Increasing antibody production coincides with decreasing virus concentrations in chicken tissues. High titres of neutralizing antibodies persist in all birds in a flock for at least 52 wk (Yuasa *et al.*, 1983b; McNulty *et al.*, 1988; Goodwin *et al.*, 1990; Hoop, 1992; Imai *et al.*, 1993). Re-infection with CAV leads to a significant increase in antibody titres (Hoop, 1992). Cardona *et al.* (2000a) reported that the combination of virus strain and genetic background of the host may influence the humoral immune response (HIR) to CAV. It has been suggested by Dren *et al.* (2000) that an early immune response induced by high dose of CAV in 6 wk old chickens prevented vertical replication and virus shedding.

So far much is not known about cell-mediated immunity (CMI) to CAV, but its role in protection against CAV cannot be ruled out (Bulow and Schat, 1997).

9.2 Passive: CAV infection in progeny of hens with antibody to CAV has not been found as maternal antibodies provide complete protection of young chicks against CAV induced disease, provided that the chicks are not immunologically compromised by other factors. Maternal antibodies are protective in chicks upto the required first 2-3 weeks of life, after which time the age related resistance develops against clinical disease but not to infection. (Yuasa *et al.*, 1980a; Imai and Yuasa, 1990; Pages *et al.*, 1997). Because of passive protection, in most of the broiler flocks only subclinical disease is produced.

10. IMMUNOSUPPRESSION

By virtue of its pathological effects, CAV is believed to be a potent immunosuppressive agent as evidenced in susceptible young chicks. The virus when transmitted by transovarian route from infected hens to progeny or following I/M administration at 1 day of age can result in a severe disease resulting in a decreased resistance and enhanced susceptibility to a wide range of viral, bacterial, and fungal infections (Rosenberger and Cloud, 1989c; Xu and Liu, 1995; Adair, 1996; Pascucci, 1997; Rosenberger and Cloud, 1998; Higgins and Warr, 2000; Jin *et al.*, 2001). Laboratory based evidence also suggest that the subclinical infections of chickens (3 wks and above) can also result in immunosuppression (McNulty *et al.*, 1991; McConnell *et al.*, 1993b; Liu *et al.*, 1995; Adair and McNulty, 1997; McNamee *et al.*, 1999; Adair, 2000; Toro *et al.*, 2000). Further indirect evidences indicate that CAV infections in older chickens are immunosuppressive which includes the increased pathogenicity of CAV, reduced vaccinal

responses to various vaccines and an apparent enhancement of pathogenicity of various co-infecting agents (Toro *et al.,* 1997; Todd, 2000).

CAV exerts a destructive effect on both primary and secondary lymphoid tissues and especially suppresses the population of both helper ($CD4^+$) and cytotoxic ($CD8^+$) T-lymphocytes in the thymus (Hu *et al.,* 1993a,b; Adair, 2000). Immunosuppression so induced is caused at least in part by apoptosis induced by VP3 protein (apoptin). Poor antibody response is observed after CAV infection at day-old age which is a consequence of depressed T_h responses in the early phase of infection (Otaki *et al.,* 1988a,b). There is marked damage of haematopoietic and lymphopoietic tissues viz. stem cells in bone marrow and precursor T-lymphocytes in thymus. The bursa, spleen and other lymphoid organs are also depleted of lymphoid cells though less severely (Goryo *et al.,* 1989a,*b*,c; Smyth *et al.,* 1993; Dhama, 2002).

Intramuscular inoculation of the virus at 1-7 days of age resulted in reduced levels of lymphocyte transformation responses of splenic, thymic and blood lymphocytes to T-cell mitogens (PHA, Con-A) and adversely affected lymphokine production activities also (IL-2, T-cell growth factor, interferon) at 7 to 21 DPI in *in vitro* studies (Otaki *et al.,* 1988a,b; Adair *et al.,* 1991, 1993a; Bounous *et al.,* 1995; Xu and Liu, 1995; Dhama, 2002). There is a decrease in the ratio between the lymphoid organs viz. thymus, bursa and spleen and the whole body weights. Also T and B cell proliferation activities decreases in immune organs resulting in a significant decrease in immunoglobulin (IgG, IgM and IgA) level in all body fluids of CAV infected chicks particularly during 7 to 35 days after infection. Thus both cellular and humoral immune functions are markedly depressed in the central as well as peripheral immune organs leading to decreased immunoprotective efficacies (Cloud *et al.,* 1992a,b; Liu *et al.,* 1995, 1997a, 2001; Zheng and Liu, 1996a, 1997, 1998; Zheng *et al.,* 1997; Dhama, 2002). Substantial reduction in macrophage functions viz. Fc receptor expression, phagocytosis, bactericidal activity and cytokine (IL-1) production have also been recorded (McConnell *et al.,* 1993a; Adair *et al.,* 1993a). Inhibition of interleukins (IL-1, IL-2 etc.) and interferon (IFN) production adversely affects molecular immunoregulatory responses on cytotoxic activities of macrophages, cytotoxic T lymphocyte (CTL), natural killer (NK) cells and expression of surface receptors.

Affected birds, if coinfected with other viruses such as MDV (Bulow *et al.,* 1986b; Zanella *et al.,* 1999; Miles *et al.,* 1999, 2001), IBDV (Yuasa *et al.,* 1980b; Rosenberger and Cloud, 1989b; Rosenberger, 1991, 1992a; Imai *et al.,* 1999), FAV (IBH/HPS) (Bulow *et al.,* 1986c; Rosenberger, 1992a; Toro *et al.,* 2000, 2001), reovirus (Bulow *et al.,* 1986c; McNeilly *et al.,* 1995), REV (Bulow *et al.,* 1986b) and NDV (De Boer *et al.,* 1994) develop a

profound immunosuppression leading to synergistic effects of both the agents (Vielitz and Landgraf, 1988; McNulty, 1991; Pope, 1991; Bulow and Schat, 1997; Markowski and Schat, 2001). Affected chickens frequently develop secondary bacterial infections with *Clostridium perfringens* and *Staphylococcus aureus* resulting into losses due to gangrenous dermatitis (Goodwin *et al.,* 1989; McNamee *et al.,* 1999). Synergism between concurrent CAV and the parasite *Cryptosporidium baileyi* in chickens have also been reported (Hornok *et al.,* 1998). Birds with mixed infections of CAV and coccidiosis were reported to have been more severely affected (Ibrahim, 1998). High doses of mycotoxins (aflatoxin) reduced paternal and subsequently maternal IgG transfer in chickens resulting in an elevated mortality and immunodepression and also possibly vertical transmission of CAV (Bulow, 1991). Co-infections can overcome the effects of age and maternal antibody related resistance, thus increasing the susceptible age period for CAV and its persistency (Yuasa *et al.,* 1980b; Rosenberger and Cloud, 1989b,c; Imai *et al.*,1999; Toro *et al.,* 2000). In the field, CAV infection seems to cause few signs of disease, however, dual infections are more serious.

CAV infection causes depression of immune response against several vaccine viruses viz. NDV, MDV, ILTV and FPV leading to vaccination failures, vaccination reactions or aggravation of the residual pathogenicity of attenuated vaccine viruses and even could lead to emergence of variant virus (Box *et al.,* 1988; Otaki *et al.,* 1988a,b; Rosenberger and Cloud, 1989c; Cloud *et al.,* 1992b; Dren *et al.,* 1992; De Boer *et al.,* 1994; Zheng and Liu, 1996b; Zheng *et al.,* 1997; Liu *et al.,* 2001; Dhama, 2002). Birds immunized against coccidiosis in the presence of CAV infection showed a lower level of protection (Ibrahim, 1998).

11. PROPAGATION OF CAV

The virus can be propagated in susceptible embryonated eggs, cell culture (MDCC-MSB1 cells) and one day-old SPF chicks or in susceptible immunosuppressed chickens. MDCC-MSB1 cells are widely used for the propagation of this virus *in vitro* (Yuasa, 1983; Yuasa *et al.,* 1983a; Bulow *et al.,* 1985). Not very specific cytopathology is observed by light microscopy but cell damage occurring after 1 to 6 subcultures is suggestive of CAV infection. CPE is not readily observed in the first few passages because noninfected MSB1 cells outgrow the infected cells. General CPE produced by the virus is characterized by inhibition of cell growth and observing enlarged, swollen and misshapen cells, cell degeneration and lysis, alkaline red medium and ultimately the inability to subculture (Yuasa, 1983; Yuasa *et al.,* 1983a; Bulow *et al.,* 1985, 1986a; McNulty, 1991; Bulow, 1991b; Dhama, 2002). Quantification is achieved by virus infectivity titrations

($TCID_{50}$/ml) by subculturing of inoculated cells every 2-3 days until cells inoculated with the endpoint dilution of CAV are destroyed (7-10 subculture may be needed) (Yuasa, 1983; Imai and Yuasa, 1990). The virus growth is generally confirmed by indirect immunofluorescent technique (IIFT) described by McNulty *et al.* (1988). CAV grows to comparatively low titres, only upto 10^5 to 10^6 $TCID_{50}$/0.1 ml in MSB1 cells.

12. POLYMERASE CHAIN REACTION

The development and application of molecular technique of PCR assay for the direct detection of CAV-DNA in infected specimens has been employed for diagnosis of CIA. CAV-DNA has been detected in various samples viz. infected MDCC-MSBI cells, chicken tissues such as unfixed liver and lymphoid organ homogenates, formalin fixed liver homogenate or formalin fixed paraffin embedded (FFPE) tissues (thymus etc.), serum and blood smears from experimentally or field infected chicks, contaminated vaccines, and in serum samples from disease free chickens (Todd *et al.,* 1992; Noteborn *et al.,* 1992b; Tham and Stanislawek, 1992a,b; Dren *et al.,* 1994; Seong *et al.,* 1996; Lauerman, 1998; Sun *et al.,* 1999a,b; Santeen *et al.,* 2001; Yilmaz *et al.,* 2001).

Primers selected on the basis of published DNA sequence of Cuxhaven-1 strain of CAV (Noteborn *et al.,* 1991) also worked efficiently with other strains from different countries indicating the region amplified was highly conserved among the strains (Noteborn *et al.,* 1992b; Tham and Stanislawek, 1992a,b; Imai *et al.,* 1998). Soine *et al.* (1993) using two pairs of primers, amplified the CAV genome of 2300 bp into two fragments of 1500 bp and 800 bp. Dren *et al.* (1994) developed a hot-start PCR, the use of a spike DNA as an internal control enabled the validation of CAV negative samples and an estimation of the number of CAV genomes present in the field samples. Rodenberg *et al.* (1994) reported that assay of CAV by PCR in avian biological products was at least as sensitive as culture in MSB1 cells and chick inoculation. Use of PCR assay with FFPE tissues is of high diagnostic value since it allows detection of both microscopic lesions and viral DNA. It also allows retrospective studies of the disease caused by or associated with CAV. The nested PCR increased the sensitivity upto 10-100 fold (Soine *et al.,* 1993; Imai *et al.,* 1998; Cardona *et al.,* 2000b). PCR based detection of CAV from blood samples might be an efficient method for diagnostic and epidemiological purposes (Rozypal *et al.,* 1997).

Kataria *et al.* (1999) detected CAV-DNA in tissues of chicks showing clinical signs of infectious anaemia by PCR. Occurrence of the disease has been long suspected in India on basis of clinical symptoms/lesions (Verma *et al.,* 1981; Goudar and Arun, 1992; Suresh *et al.,* 1995) or reported by viral antigen detection by IPT (Venugopalan *et al.,*

1994). By employing PCR, transmission studies and virus isolations Kataria *et al.* (1999) confirmed the occurrence of CIA in India. Subsequently, Dhama (2002) utilized PCR for diagnosing CAV infection in clinical samples/experimental studies and its detection during *in vitro* isolations and passages in MSB1 cells. Cardona *et al.* (2000b) employing nested PCR reported wide distribution and longer persistence of CAV in the reproductive tissues of infected birds. Yamaguchi *et al.* (2000) established a rapid and highly reproducible, sensitive and reliable quantitative method for infectivity titration of CAV by competitive PCR possessing several advantages over the conventional methods. Recently, Markowski *et al.* (2002) developed a strain-specific real-time PCR for Quantitation of CAV.

PCR is very effective for the early detection of CAV in cell lines or infected tissues of the affected birds or detection of subclinical CAV infections, screening of SPF flock, or testing CAV contamination of cell lines, virus preparations and preferably poultry vaccines. It is expected that the PCR assay will offer a highly efficient replacement of current laboratory tests for CAV diagnosis and research and for the study of molecular epizootiology of CIA.

13. SEROLOGICAL ASSAYS

Detection of antibodies is more reliable for ascertaining whether the flocks have been exposed to CAV but do not determine if the birds are infective or even non-indicators of current infection status (Cardona *et al.,* 2000a). These are important for sero-monitoring of SPF and breeder flocks for CAV specific antibody to make sure that they have sero-converted so as to avoid the risk of CAV contamination of avian vaccines and clinical disease in progeny flocks, respectively. Antibodies to CAV in chicken sera or egg yolk can be assessed by virus neutralization (VN), enzyme linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF) and immunoperoxidase (IIP) tests (Bulow *et al.,* 1985; Bulow and Fuchs, 1986a; McNulty *et al.,* 1988; Todd *et al.,* 1990b; Brewer *et al.,* 1994). The commercially available ELISA kit and IIF testing are commonly used to monitor SPF chickens for CAV infection.

13.1. Indirect Immunofluorescence Technique (IIFT): Indirect immuno fluorescence technique is usually employed for survey of antibody against CAV or monitoring the infection effectively in SPF and commercial chicken flocks (McNulty *et al.,* 1988: Chettle *et al.,* 1991; Lamichhanne *et al.,* 1992; Fadly *et al.,* 1994; Dren *et al.,* 1996; Zhou *et al.,* 1996). IIFT has been effectively employed for screening of hybridoma supernatent fluid (McNulty *et al.,* 1990c). CAV infected MSB1 cells are used as positive antigen for detecting antibody to CAV. IFA titres of 1:40 and higher are generally considered positive. Although it has been conventionally used in most serological surveys for breeder flocks (McNulty,

1991), the test however, lack sensitivity in detecting low levels of antibody to CAV as compared to SN, and is prone to non-specific or false positive results (Bulow, 1988; Lucio and Schat, 1990; Otaki *et al.,* 1991; Hoop, 1992). Use of serum dilutions at 1:40-1:100 or even more in the IIFT to avoid non-specific staining or prozoning effects has been recommended. Employing MAbs against VP3 (CAV specific protein) infected cells can also be efficiently stained in an IIFT (Todd *et al.,* 1990a; Noteborn *et al.,* 1994a; Renshaw *et al.,* 1996; Brown *et al.,* 2000).

13.2 Indirect Immunoperoxidase Test (IIPT): The IIP assay also utilizes CAV-infected MDCC-MSB1 cells as antigen for detecting antibody to CAV (Lamichhanne *et al.,* 1992). Some non-specific reactions are observed as with that of IIFT. IIPT was found to be at least as sensitive as the IIFT but less sensitive to ELISA (Chettle *et al.,* 1991; Lamichhane *et al.,* 1992; Toro *et al.,* 1994a,b).

13.3 Enzyme Linked Immunosorbent Assay (ELISA): Development and use of ELISAs to detect and measure CAV antibodies have been studied by several workers (Todd *et al.,* 1990b, Otaki *et al.,* 1991; Goodwin *et al.,* 1992c; Pallister *et al.,* 1994; Iwata *et al.,* 1998; Todd *et al.,* 1999). Todd *et al.* (1990b) developed an indirect ELISA employing CAV specific monoclonal antibodies, 4H4, to capture partially purified viral antigen selectively. Highly purified CAV also has been found to be a suitable ELISA antigen (Goodwin *et al.,* 1992c; Lamichhane *et al.,* 1992) but the use of more purified preparations is not economically viable. The ELISA plates directly coated with partially purified viral antigen have also been found equally suitable (Brewer *et al.,* 1994).

Commercial CAV ELISA kits employing the above methods have been developed and are available commercially (Flockscreen CAV antibody ELISA kit, Guildhay Ltd., UK; CAV antibody test kit, IDEXX Laboratories, Inc., Westbrook, ME, U.S.A.). Clarified lysates of infected MSB1 cells have been used as ELISA antigens (Kling, 1991; Otaki *et al.,* 1991) but appear less suitable than purified or semipurified virions for routine testing. Pallister *et al.* (1994) developed indirect capture ELISA with MAb for detection of serum antibody to CAV based on cloned capsid protein (VP1). Iwata *et al.* (1998) reported the use of recombinant VP2 and VP3 as antigen to detect anti-CAV antibodies in ELISA. Later, Todd *et al.* (1999) developed a blocking ELISA, having several advantages, utilizing CAV specific MAb (2A9) (McNulty *et al.,* 1990c) which reacted with geographically different field isolates indicated worldwide application of ELISA test. On the basis of speed and cost and being more suitable for testing large numbers of serum samples, ELISA is the test of choice for investigating the epidemiology of CAV in poultry (Todd *et al.,* 1999).

But ELISA has got its own limitations viz. obtaining enough antigen due to inability of virus to grow to high titres *in vitro* and non-specific reactions with anti-CAV polyclonal chicken antibodies (O'Rourke *et al.*, 1994; Michalski *et al.*, 1996). False positive reactions reported in upto 18% of sera from SPF chicken were found to be associated with the transient presence of particular protein fraction (O'Rourke *et al.*, 1994). Therefore, only highly purified and concentrated preparations of CAV are suitable for detecting antibodies by ELISA and western blot techniques. It has been suggested that antigen capture/blocking ELISAs employing MAbs and developing recombinant (r) CAV proteins (antigens) can alleviate these limitations (Todd *et al.*, 1990b, 1994, 1999).

14. PREVENTION AND CONTROL

Control measures are mostly directed at limiting vertical transmission and subsequent clinical disease outbreaks by the use of vaccine in parent flocks several weeks before egg production to ensure that breeder flocks have seroconverted before coming into lay. Sound management, hygiene and strict biosecurity practices will be of immense help in preventing young chicks from early exposure to CAV as well as coinfections with other lymphocidal agents especially IBDV and MDV following suitable vaccination programmes, so as to limit immunosuppression and reduce the economic losses (McIlroy, 1994; Jorgensen *et al.*, 1995a,b; Van Den Berg, 1996; Fussel, 1998; Engstrom, 1999; De Herdt *et al.*, 2001).

Acquired immunity efficiently prevents vertical transmission in the flock. The effective approaches followed included controlled exposures by artificial litter transfer, autogenous vaccine comprising of affected tissue (particularly liver) homogenates and a live wild-type virus vaccine administered in drinking water to parent flocks during the rearing period, but are risky and cannot be recommended as standard procedure due to hygiene problems, risk of immunosuppression in young chicks, doubtful CAV concentration and co-existance of other pathogens (Vielitz *et al.*, 1989; McNulty, 1991). Live vaccine comprising of chicken embryo propagated Cux-1 isolate using $10^{4.5}$ $TCID_{50}$/bird although gave efficient protection but harboured risks in practice of reversion to virulence (Vielitz *et al.*, 1989; Vielitz, 1989a,b; Rosenberger, 1992b). A successful commercial live vaccine comprising of non-attenuated chicken embryo propagated virus to be administered through the drinking water has been developed (Vielitz and Voss, 1994). But these approaches may not be acceptable in all countries due to risk involved in the use of non-attenuated virus. Steenhuisen *et al.* (1994) developed an attenuated CAV live vaccine by serial passages in chicken embryo, that has to be administered via parenteral routes to be fully protective.and with an adjuvant result in a better humoral immune

response in the vaccinated birds (Cardona *et al.*, 2000a). Cell culture propagated CAV, given multiple passages in MSB1 cells, can also serve as an effective drinking water vaccine provided that the titre is adequate. It, however, cannot have much utility as fully attenuated vaccine because reversion to virulence even after many passages (P170) have been reported, and that MSB1 cells are productively infected with MDV (Todd *et al.*, 1995, 98). Classical live vaccines against CAV, therefore, possess residual pathogenicity and a risk of reversion to virulence.

Vaccination with live vaccines should be performed at about 13-15 wk of age, 3-4 weeks prior to the onset of lay to avoid the hazard of spreading vaccine virus through the egg (Vielitz *et al.*, 1987; McNulty, 1991). Regular sero-monitoring is desired for formulating prevention strategies or to test the efficacy of vaccinations.

Pages *et al.* (1997) developed an oil emulsified formalin inactivated CAV vaccine from MSB1 pool of CAV which proved to be highly immunogenic and protective against the disease in most of the progeny of vaccinated breeders upto the first 3-4 wks of life. Possibility of reversion to virulence is also ruled out.

Conventional approaches for development of a vaccine present difficulties due to the inability of the virus to grow to high titres in embryos/cell cultures (*in vitro*) and the unavailability of naturally occuring apathogenic isolates of CAV. Alternatively, by development of immunogenic recombinant proteins to produce a sub-unit vaccine and/or a live attenuated/recombinant (r) virus vaccine through the manipulation of the virus DNA, will serve the purpose. Koch *et al.* (1995) developed an effective sub-unit vaccine using recombinant baculovirus insect cell system synthesizing and expressing CAV proteins VP1 and VP2 simultaneously. It provided an attractive and safe alternative to wild type CAV for vaccination in chickens without undue risk, and also the problem of obtaining satisfactory levels of CAV antigen at an acceptable price might be overcome.

Scott *et al.* (1999) reported a highly passaged (P310 in MSB1 cells) and cloned CAV to be substantially attenuated which can serve as an effective aid for the development of a live CAV-vaccine. It may also be possible to produce attenuated isolates by rational design, for example Noteborn *et al.* (1998b) have reported that mutations on the 12 nt sequence contained within the array of 19 nt repeats, associated with enhancer-promoter activity, reduces virus replication and cytopathogenicity in cell culture, and the mutant CAV strains were able to produce a neutralizing conformational epitope, implying that they can trigger the required immune response, making these as potential candidates for the development of an attenuated CAV vaccine, though, its attenuation level remaind yet to be tested. Recent report of Yamaguchi *et al.* (2001) highlighted the single AA

change at residue 394 of the VP1, major determinant of pathogenicity, by cloning and passage in MSB1 cells or point mutation can generate a low pathogenic CAV which is a good candidate for developing a genetically homogenous, safe and stable CAV live vaccine. Similar approaches of designing attenuated isolates needs attention. The development of CAV vaccine will perhaps help control not only CIA but also other diseases that are aggravated by CAV.

15. NUCLEIC ACID IMMUNIZATION

Immunization or vaccination is called 'the third generation vaccine' methodology for achieving specific immune activation (Weiner and Kennedy, 1999). The concept of DNA as a vaccine was initially described in 1990 in a seminal study by Wolff *et al.* (1990) demonstrating that direct intra-muscular injection of purified bacterial plasmid DNA resulted in the expression of an encoded reporter gene. This study provided a strong basis for the concept that 'naked DNA' can be delivered *in vivo* and can direct protein expression for initiating an immune response (Ulmer et al., 1993). The gene encoding the immunogenic protein(s) is inserted into an appropriate eukaryotic expression plasmid that can be replicated in bacteria, purified and then directly inoculated by various methods into the animal to be vaccinated. The recent review of nucleic acid immunization has covered all the aspects and highlighted the issues to be addressed in chicken (Oshop *et al.,* 2002b).

15.1. Immunology of DNA vaccines: The exact mechanism of how DNA vaccines initiate an immune response remains unknown, but there are several theories as to how this may occur. One single injection of a plasmid encoding foreign genes can initiate a broad spectrum of immune responses (Wolff *et al.,* 1990; Kowalczyk and Ertl, 1999). The quantity of antigen produced after DNA inoculation is usually in the picogram to nanogram range. Despite this, there is an efficient induction of a sustained and broad-based immune response, which might be due to the immune-enhancing properties of the DNA itself (i.e. CpG motifs) and /or the type of antigen presenting cell (APC) transfected. Since the peptides are recognized as being foreign, they are then processed and presented to the host immune system initiating either numeral, cell-mediated immunity, or both. This process of antigen presentation is similar to that occurring during a natural infection, since the proteins are presented to the immune system in their native form. This represents one of the most important features of DNA vaccines; the ability to induce humoral and cell-mediated immunity through the priming of CD4+ and CD8+ cells (Gurunathan *et al.*, 2000).

15.2 Delivery-route, dose and regime: Depending upon the route of delivery and the age of the bird, a wide range of DNA concentrations have been administered to the avian in an effort to induce a measurable response (immunity or protection). In a single injection, the least amount was 0.25μg per bird given by ballistic delivery (Kodihalli *et al.,* 1997) with the most being 750 μg per bird given by IM injection (Triyatni *et al,* 1998). As a total given per bird over time the amount of DNA administered ranged from 0.5 to 1500 μg (Kodihalli *et al.,* 1997; Triyatni *et al.*, 1998). In studies where different doses were used a clear dose related response was found (Suarez and Schultz-Cherry. 2000). Interestingly, in the work by Suarez and Schultz-Cherry (2000) a limiting amount of DNA was found to be best (100 μg per bird by IM injection) beyond, which the antibody response was diminished.

15.3 Immunity and protection from challenge: The published reports of nucleic acid immunization in poultry have shown an immune response in the form of humoral, cellular or protective immunity. Although a few DNA vaccine studies in the avian have failed to show a measurable antibody response, most have been able to demonstrate immunoglobulin production. In those studies where no antibody was found, the vaccinated birds were still partially to fully protected from challenge, indicating that an adaptive immune response other than humoral (presumably cellular immunity) was induced by the vaccination (Kodihalli *et al.,* 1997, 2000; Fynan *et al.,* 1993). In the studies where antibody was found the primary isotype was IgG (Vanrompay *et al.,* 1999b, 200l) or IgY (in the studies where yolk antibodies where found) (Rollier *et al.*, 2000a: Romito *et al.*, 2001) and the antibodies were found to be functional, in that they neutralized haemagglutination or infectivity of viruses in serum neutralization assays (Triyatni *et al.,* 1998; Rollier *et al.*. 2000a; Suarez and Schultz-Cherry, 2000)

Very few studies have been done detailing the cellular immune responses to DNA vaccination in the avian, however many reports have suggested that this arm of the immune system is activated. The most conclusive study was by Seo *et al.* (1997) who specifically assessed the activity of cytotoxic lymphocytes after DNA immunization. This study reported that a plasmid encoding a CTL epitope of infectious bronchitis virus induced CD8+ cell-mediated killing of infected targets and that these immunized chickens were protected from acute viral infection. Other studies have shown specific activation of lymphocytes or changes in lymphocyte subpopulation after DNA vaccination, but have not measured the activity of cytotoxic lymphocytes (Vanrompay *et al.*, 1999, 2001; Song *et al.,* 2000)

15.4 *In ovo* immunization: Neonatal immunization of animals with DNA vaccines has been done and shown to work effectively (Wang *et al.*, 1997; Siegrist, 1997, 2001; Pertmer and Robinson, 1999; Hassett *et al.*, 2000). In addition, DNA vaccination of the neonate has been shown to overcome maternal immunity, unlike other protein based vaccines (Hassett *et al.*, 1997; Oshop *et al.*, 2002a). Johnson *et al.* (1997) showed with a reporter gene that IM inoculation of the embryo was possible and that protein was made. A modified version of this *in ovo* technique to deliver nucleic acid vaccines to the embryo was developed (Oshop *et al.*, 2002b). This modified in ovo delivery technique involves combining plasmid DNA enveloped in a neutral lipid along with an equal part of dimethyl sulfoxide (DMSO) and depositing the mixture through a hole in the top of the eggshell onto the shell membrane.

16. CAV-VP3 AND ITS ANTI - NEOPLASTIC EFFECT

Chicken anaemia virus induces apoptosis in thymus of infected chicks and *in vitro* infected MDCC MSB-1 cells (Jeurissen *et al.*, 1992). Later, it was found that VP3 protein of CAV alone could induce apoptosis (Noteborn *et al.*, 1994). It has been proven by cloning of the gene coding for VP3 protein in plasmid and transfecting in MDCC-MSB-1 cells, which showed induction of apoptosis and necrosis by propidium iodide staining of cell nucleus and DNA laddering pattern. It has been observed that VP3 could not induce apoptosis in primary chicken embryo fibroblast cells. It was also reported that apoptin induced apoptiosis in various human transformed and/or tumerogenic cell lines, but not in normal cells. The c-terminally 11 amino acids truncated protein was unable to induce apoptosis (Noteborn *et al.*,1994). It was found to be unsuitable for its usage as antigen or immunogen (Cunninghom *et al.*, 2001).

In normal cells, P^{53} is the essential intracellular mediator of apoptosis apart from the death receptor. These pathways dismantle the cells finally by activation of caspase proteases. Zhuang *et al.* (1995) first screened this protein in three inbreed mice lines of P^{53} mutant, P^{53} normal and P^{53} deficient to study the role of P^{53} in apoptin mediated apoptosis and concluded that apoptin induced apoptosis independentiy in all the three mice lines.

Later, Danen van Oorschot *et al.* (2000) have analyzed the role of caspases in apoptin-induced apoptosis using a specific antibody, active caspase-3 was detected in cells expressing apoptin and undergoing apoptosis. They concluded that apoptin employed cellular apoptotic factors for induction of apoptosis, although activation of

upstream caspases was not required but activation of caspase-3 and possibly other downstream caspases were essential for rapid apoptin induced apoptosis.

Pieterson and Noteborn (2000) have also reported that tumor cells were resistant to apoptosis induced by cytotoxic agents than normal cells, however, the resistance could be a direct consequence of mutations in certain tumor suppressor genes (P^{53}) or of certain proto-oncogens (Bcl-2). Apoptin acted independently of P^{53} was stimulated by Bcl-2 , and insensitive to BCR-ABL which meant that apoptin induced apoptosis in cases where current therapeutic agents would fail.

Apoptin induces apoptosis specifically in tumor cells and not in normal cells. It is proven to induce cell death by apoptosis in various cell lines derived from tumors eg. hepatomas, lymphomas, leukemia, melanomas, breast and lung tumors and colon tumor. It could not induce apoptosis in normal cells viz. human keratinocytes, vascular endothelial cells, smooth muscle cells, human primary T cells, human diploid fibroblasts as well as murine and rat embryonal fibroblast. The possible reason could be that apoptin is located in the nucleus and is able to perform its activity in tumor cells. In normal cells, it could not do so since it is located in specific cytoplasmic structures.

In vivo effects of apoptin have also been tested by constructing recombinant adenovirus expressing apoptin. It was found that it could be administered safely by single or repeated Intravenous (i.v) injections without any severe adverse effects. It was also seen that a single intratumoral injection of this recombinant adenovirus into a xenogenetic hepatoma in nude mice showed a reduced tumor growth (Pietersen *et al.,* 1999). Recombinant Parvovirus vector expressing apoptin was also adjudged to induce anti-neoplastic effect (Olijslagers *et al.,* 2001).

LeLiveld *et al.* (2003a) showed that recombinant apoptin protein spontaneously formed non-covalent globulin aggregates comprising 30-40 subunits *in vitro.* This multimerization was robust and virtually irreversible, and the globular aggregates were also stable in cell extracts suggesting that they remained intact within the cell. Their study also indicated that covalently fixing the apoptin monomers within the recombinant protein multimer by internal cross linking did not affect the biological activity of apoptin, as these fixed aggregates exhibit similar tumor specific localization and apoptosis inducing properties as noncross-linked apoptin.

Zheng *et al.* (2003) also showed that formation of uniform distinct, stable multimeric complex inside the cell on cytoplasmic microinjection. Their further studies revealed that recombinant mannose binding protein -apoptin multimers retain its biological activity similar to the ectopically expressed wild type apoptin; namely the complexes

translocated to the nucleus of the tumor cells and induced apoptosis, whereas they remained in the cytoplasm of normal primary cells and exerted no apparent toxic effect.

LeLiveld *et al* (2003b) later reported that apoptin predominantly co-localized with heterochromatin and nucleoli within tumor cells on the basis of immunoelectronmicroscopy. They have also reported that apoptin co-operatively forms distinct superstructures with DNA *in vitro* and these superstructures do not go beyond 20 multimeric apoptin complexes and approximately 3 Kb of DNA. Further, they have showed that a single apoptin multimer to have eight independent nonspecific DNA binding sites which predominantly bind strand ends. Apoptin also have high affinity for naked, uncoated double and single stranded DNA since this form of DNA are predominantly found in transcriptionally active.

Perusal of the literature indicated the need of characterization of this emerging virus prevalent in India and to know the exact epidemiological status of this disease in the subcontinent. Also, a safe, highly effective and potent vaccine is lacking for the effective prevention and control of this disease in the field. The apoptotic potential of the CAV-VP3 protein has to be exploited to develop a safe anti-neoplastic drug for various kinds of neoplasm.

MATERIALS AND METHODS

1. VIRUSES

Five Indian field isolates of CAV and Rous sarcoma virus (RSV) used in the present study were maintained in the Division of Avian Diseases, IVRI, Izatnagar. All the five CAV isolates were isolated from the materials submitted for disease investigation from different regions of the country. Details of these CAV isolates are given in Table 1.

2. CELL CULTURE

CAVs were propagated in Marek's disease virus transformed (MDCC-MSB-1) cells. The cell line was maintained and used following the method described by Goryo *et al.* (1987b). Cells were cultured in RPMI-1640 growth medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, USA) at 39°C in an incubator (Nuaire, USA) with humidified atmosphere containing 5% CO_2. Cells were maintained by subculturing at 2-3 days intervals. RSV was grown in primary chicken embryo fibroblast (CEF) cell culture prepared from the 11-12 day old embryos.

3. CHEMICALS

All the chemicals and enzymes used in this study were procured from Sigma, MBI Fermentas, Qiagen, Invitrogen, Banglore Genei and Sisco Research Laboratory.

4. PLASTIC WARES

All the plastic wares used in this study including tissue culture flasks, plates, eppendorf tube, micropipette tips, PCR tubes and filtered tips were procured from different reputed firms viz. Corning (USA), Axygen (USA), Nunc (Denmark) and Tarson (India).

5. GLASS WARES

The glasswares used during the study were procured from Borosil (India) and Duran (Germany). These were thoroughly washed and sterilized for tissue culture work as per standard procedure.

6. EMBRYONATED CHICKEN EGGS AND CHICKENS

Specific pathogen free (SPF) embryonated eggs, certified free from various poultry pathogens, were kindly supplied by M/S Venkateshwara Hatcheries Group Limited (VHL), Pune. These eggs were incubated in an isolated setter and hatcher at the Hatchery Unit of Central Avian Research Institute (CARI), Izatnagar. White leghorn layer birds were obtained from the experimental layer farm of the same Institute.

Table 1. Details of field isolates of CAV used in the present study

Sl. No.	Place of Isolation (States)	Divisional No. of isolates	Code No.
1.	Pune, Maharashtra	CAV, AD 501a/2001	CAV-A
2.	Noida, Uttar Pradesh	CAV, AD 498/2000	CAV-B
3.	Gurgaon, Haryana	CAV, AD 172/1994	CAV-C
4.	Palampur, Himachal Pradesh	CAV, AD 505/2001	CAV-E
5.	Namakkal, Tamil Nadu	CAV, AD 38/1993	CAV-P

7. BUFFERS AND REAGENTS

The details of buffers and reagents used in this study are given in Appendix.

8. CULTIVATION OF THE VIRUS

During the initial adaptation, virus inoculum (0.5ml) prepared from the tissue material was mixed to the cell pellet suspended in 0.5ml of the medium and incubated at 39^0C for 1 hour in a 15 ml centrifuge tube. Then, 5 ml of the RPMI medium was added into the tube, cells were suspended in the media, seeded into culture flask and incubated at 39^0C with 5% CO_2 concentration in a humidified atmosphere for 72 hr. After 72 hr of incubation, 1 ml of the cell suspension was transferred into 4 ml of fresh medium and incubated. The process was repeated 2-3 times to appreciate the cytopathic effect and virus was harvested in every passage.

9. TITRATION OF CAV

Quantification of the CAV isolate A passaged 4 times in MSB1 cells was done as per the method of Imai and Yuasa (1990). Titration was carried out in MSB1 cell cultures using 96-well tissue culture plates. Briefly, serial ten-fold dilutions (10^{-1} to 10^{-7}) of the virus were prepared in RPMI-1640 without serum. 50 µl aliquots of these dilutions were transferred into the culture wells followed by adding $4X10^4$ MSB1 cells in 150 µl culture medium to each well. Each dilution was tested in five concurrent cultures with eight passages at 3-day intervals. Cells were subcultured at 2-3 days intervals by adding 50 µl of the infected cell suspension into 150 µl RPMI growth medium. Cultures without virus were included as controls. Infectivity endpoints were based on the cytopathic effect, performing subculture until degenerative changes developed in the control set (Yuasa, 1983). Mean tissue culture infective dose ($TCID_{50}$) was calculated according to the method of Reed and Muench (1938).

10. EXTRACTION OF VIRAL DNA

Viral DNAs of CAV isolates were extracted from the infected MSB1 cells at each passage following the method as described by Tham and Stanislawek (1992a) with some modifications. Procedure included lysis of infected cells, deproteinization by phenol and chloroform extraction and finally precipitation with ethanol. The steps involved were as follows:

1. CAV inoculated MSB1 cells were harvested at 48 hr PI in quantity of 1.5 ml and centrifuged at 3,000 rpm for 10 min at room temperature. Supernatant was discarded carefully leaving 100 µl of supernatant with MSB1 cell pellet.

2. Pelleted MSB1 cells were lysed by treating with 900 µl of lysis buffer (10mM Tris, 10mM EDTA, 0.25% Triton X-100), vortex mixed gently and kept for 10 min at room temperature
3. Subsequently, 40 µl of 5 M stock NaCl (0.2 M final concentration) was added, mixed gently and centrifuged at 3,000 rpm for 10 min at 4^0 C for precipitating cellular/high molecular weight DNA.
4. 585 µl of the above supernatant was collected, reacted with 15 µl of 20 mg/ml proteinase-K (0.5 mg/ml final concentration) and incubated at 37^0 C for 3 hr.
5. Subsequently, the lysate was extracted with standard phenol : chloroform (1:1) and chloroform treatment.
6. 360 µl of the acquous phase was collected and precipitated with $1/10^{th}$ volume of 3M sodium acetate and 2.5 volume of ethanol at -20^0C.
7. The DNA was precipitated by centrifugation at 12,000 rpm for 10 min and washed once with 70% ehtanol. The pellet was air-dried and dissolved in 30 µl of nuclease free water.

Uninfected MSB1 cells were similarly treated for DNA extraction.

11. SEQUENCE ANALYSIS OF THE CAV GENOME

A strategy was designed for the sequencing of the complete genome of CAV and to get the products of CAV genes for the cloning and expression purposes. The strategy is given diagrammatically in Fig 1. The details of primers used to amplify different regions of the CAV genome is given in Table 2. Totally, four sets of primers were used. Primer set #1 was used to amplify the VP1 region and Primer set #2 was used to amplify the VP2 region. These two sets covered the entire coding regions of CAV. The primer set #3 was used to amplify the noncoding region of the CAV. The PCR amplification was carried out in PCR buffer containing 1.5mM $MgCl_2$, 200 µM of each dNTPs, 10 pmoles of each primer, and 1.0 Unit of *Taq* polymerase in 25 µl of total reaction volume. The reaction was carried out in an automated thermal cycler (PTC 200, MJ research, USA).

11.1 PCR amplification of VP1 region

Amplification of the VP1 region was carried out using the primer set #1 (VP1F & VP1R) with initial denaturation of 94°C for 4 min followed by 34 cycles of denaturation, annealing and extension at 94 °C /1 min, 57 °C /1 min and 72 °C /2 min, respectively, and the final extension was carried out at 72 °C /8 min. The standardized PCR amplification yielded a distinct band of 1390 bp in size as expected.

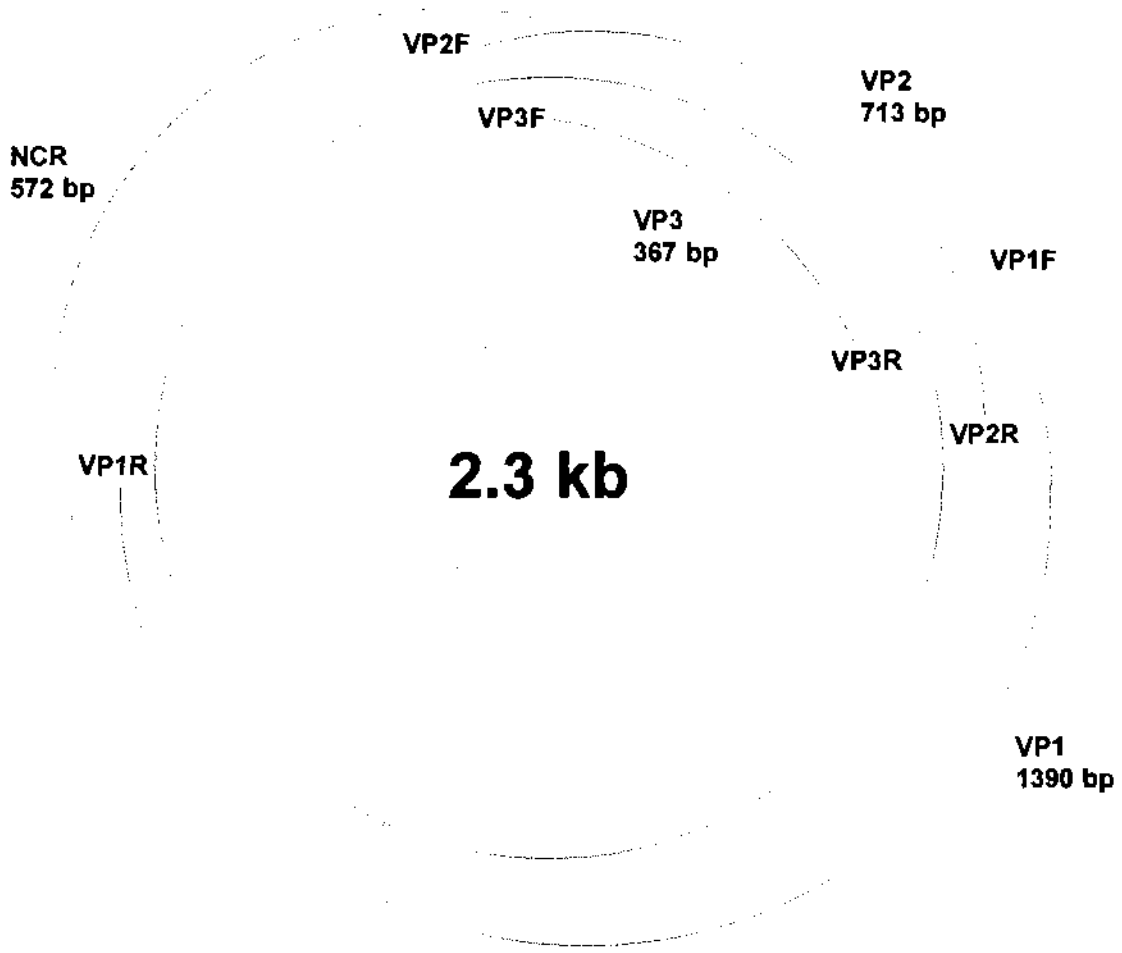


Fig. 1: Diagrammatic representation showing the position of the primers and amplification of different regions by PCR in the present study

Table 2. Description of the self designed primers used in the study

Primer Set	Primer	Mers	Primer Sequence (5'- 3')	Amplicon size
#1	VP1F	22	AGC CGA CCC CGA ACC GCA AGA A	1390
	VP1R	23	TCA GGG CTG CGT CCC CCA GTA CA	
#2	VP2F	21	GCG CAC ATA CCG GTC GGC AGT	713
	VP2R	23	GGG GTT CGG CAG CCT CAC ACT AT	
#3	NCRF	20	GGA CGT CAA CTG GGC GAA CA	572
	NCRR	20	CCG CCG TTC CCG TGC ATC CA	
#4	VP3F	19	ATG AAC GCT CTC CAA GAA G	367
	VP3R	20	CTT ACA GTC TTA TAC ACC TT	

11.2 PCR amplification of VP2 region

Amplification of the VP2 region was carried out using the primer set #2 (VP2F & VP2R) with initial denaturation of 94°C for 4 min followed by 34 cycles of denaturation, annealing, extension at 94 °C /1 min, 63 °C /1 min, 72 °C /1 min, respectively and final extension was carried out at 72 °C /5 min. The standardized PCR amplification yielded a distinct band of 713 bp in size as expected.

11.3 PCR amplification of the Noncoding region of CAV

Amplification of the noncoding region was carried out using the primer set #3 (NCR-F & NCR-R) with initial denaturation of 96°C for 3 min followed by 34 cycles of denaturation, annealing and extension at 96 °C /1 min, 54 °C /1 min and 72 °C /1 min, respectively, and the final extension carried out at 72 °C /5 min. The standardized PCR amplification yielded a distinct band of 572 bp in size as expected.

11.5 Sequencing of the PCR products

The PCR amplified products of different isolates were sequenced in the automated sequencer (ABI Prism, Japan) by employing the big dye termination kit. Single reaction comprised of ready reaction mix (2.5X) – 4 µl, big dye sequencing buffer (5X) – 2 µl, primer – 3.2 pmoles, template DNA - 5 µl and nuclease free water upto 20 µl. The reaction was carried out in a thermal cycler (Eppendorf, USA) for a total number of 50 cycles. The cycle steps followed were 94°C/4 min (initial denaturation), 94°C/30 sec (cyclical denaturation), 55°C/10 sec (cyclical annealing) and 60°C/4 min (cyclical elongation). After completion of the reaction, the amplicon was purified. EDTA (5 µl) and ethanol (100 µl, room temperature) were added to the reaction mix (20 µl) directly and mixed well. The mixture was kept at room temperature for 15-30 min. Later, it was centrifuged at 12,000 rpm for 20 minutes and the supernatant was discarded carefully. The pellet was washed once with 70 µl of the 70% ethanol and dried. Hi-Di formamide was added to the pellet and mixed well by gentle vortexing. After a short spin, the mix was denatured at 95°C for 2 min and chilled on ice immediately for 5 min. The sample was loaded in the automated sequencer (ABI prism, Japan) and the sequence was obtained.

11.6 Sequence alignment

Using EditSeq and MegAlign programme of Lasergene Software (DNASTAR Inc., USA), all the sequences obtained were aligned by Clustal method. The nucleotide sequences obtained were also translated into amino acid using EditSeq programme and aligned by Clustal method. For comparisons, the nucleotide and amino acid sequences of

known reference strains were included. These sequences were downloaded from the national center for biotechnological information (NCBI) web site. Accession numbers and other details of these viruses are given in Table. 3. Percent divergence and percent similarities based on the nucleotide and amino acid sequences could also be drawn using MegAlign programme.

11.7. Phylogenetic analysis

Aligned nucleotide and amino acid sequences were subjected to phylogenetic analysis on Megalign programme of Lasergene software. Phylogenetic tree (cladogram) was drawn with unbalanced branches showing branch length proportionate to sequence divergence.

12. PATHOGENICITY STUDIES OF INDIAN ISOLATES OF CAV

Day-old SPF chicks (n=60) were divided into six groups. Each group comprised of 10 chicks. Group 1 was kept as control and groups 2-6 were infected with each CAV isolate separately. The groups were maintained under strict isolated conditions and high biosecurity measure was taken to avoid the cross infection. The CAV isolates were infected at a dose rate of 1 ml cell culture fluid ($10^{4.5}$ $TCID_{50}$/ 0.1ml) per bird intramuscularly in thigh region. The chicks of all the groups were observed daily for clinical signs and mortality, and PCV and body weight gain were recorded on 5, 10 and 15 days post infection. At 15^{th} day, all the birds were sacrificed and gross lesions were recorded in organs viz., thymus, bone marrow, spleen, liver and bursa. The same organs were collected for histopathological analysis. The data obtained were analyzed statistically, wherever needed.

13. MOLECULAR CHARACTERIZATION OF CAV VP1 AND VP2 PROTEINS

The antigenic effect of CAV VP1 and VP2 was studied by cloning and expressing the proteins in suitable eukaryotic system and the immunogenic and protective effects were studied in chickens through nucleic acid immunization approach.

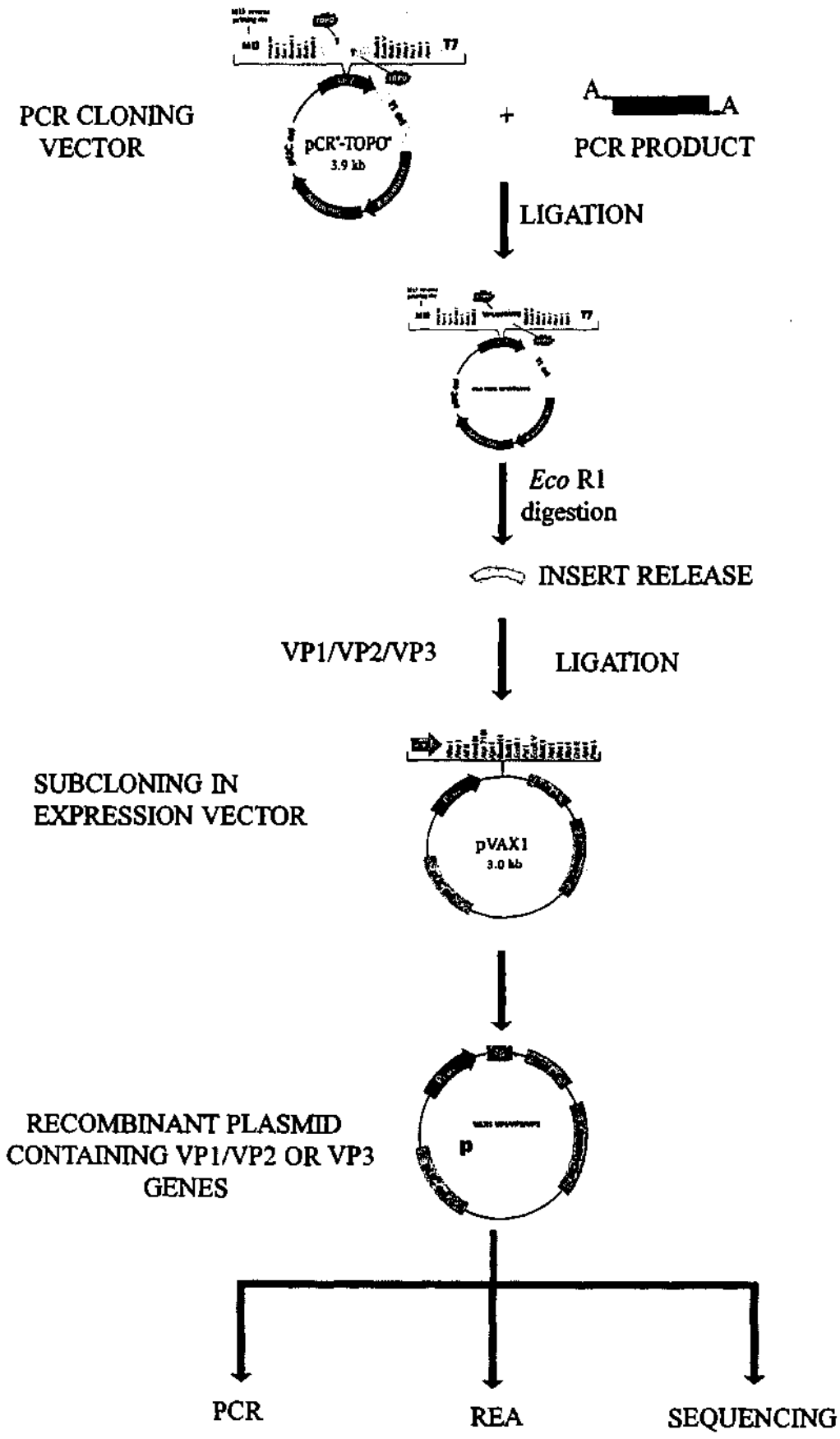
13.1. Cloning of CAV-VP1 and VP2 in pVAX vector

The cloning strategy used for the cloning of CAV-VP1 or VP2 genes is depicted in Fig. 2. The PCR amplified, gel purified (using Qiex II gel extraction kit, Qiagen, Germany) VP1 and VP2 were initially cloned in TOPO-T vector (Invitrogen, USA). The insert was released by digesting with *Eco* RI restriction enzyme and the released product was purified from the gel. It was ligated into the linearised pVAX expression vector using the same restriction enzyme and dephosphorilated. The ligation mixture was used to transform the one-shot competent cells (Invitrogen, USA). The colonies obtained were screened for the

Table 3. Description of the CAV Strains with EMBL accession numbers used in the present study for comparison.

S.No.	Isolate/Strain	Origin	Accession No.
1.	Cux-1	Germany	M55918
2.	26P4	Netherlands	D10068
3.	704	Australia	U65414
4.	BD-3	Bangladesh	AF395114
5.	SMSC-1	Malaysia	AF285882
6.	SMSC-1P60 (attenuated)	Malaysia	AF390102
7.	Harbin	China	AF475908
8.	Del Ross	USA	AF313470
9.	98D02152	USA	AF311892
10.	A2	Japan	AB031296
11.	TR-20	Japan	AB027470

CLONING STRATEGY



right orientation by PCR using the T7 promoter primer of vector and reverse primer of the insert. The clones gave the positive amplification were considered to have right orientation of the insert. Singe PCR positive clone was grown in 200 ml of the LB media and the plasmid was purified using plasmid midi prep kit (Qiagen, Germany). The purified plasmid was used for the sequencing using the T7 promoter sequencing primer to further confirm the right orientation and presence of gene start, and for the *in vitro* expression studies.

13.2 *In vitro* expression studies

Hep 2 cells showing 50% monolayer at 20 hours in 6 wells culture plate was used for the expression study of the pVAX-CAV-VP1 plasmid. The *in vitro* expression of pVAX-CAV-VP2 was studied in chicken embryo fibroblast cells. The monolayer cells were transfected as described below.

1. 6 µg of the plasmid and 12 µl of the lipofectamine plus reagent were mixed in 100 µl of the OptiMEM media in a 1.5 ml sterile microcentrifuge tube and allowed to stand in room temperature for 15 minutes.
2. 8 µl of the lipofectamine was also mixed in 100 µl of the OptiMEM and kept in room temperature for 15 minutes.
3. The contents of the above mentioned two steps were mixed together and kept at room temperature for 15 minutes.
4. The monolayer in the 6 well plate was washed with sterile PBS and 2 ml of growth media containing 5% FBS was added.
5. Later, 800 µl of the OptiMEM was added dropwise to the mixture on step 3, it was mixed well. The mixture was added to the plate dropwise and incubated in a incubator at 37^0 C with 5% CO_2 tension.
6. After 4 hours of incubation, the media over the cells were removed and fresh media was added.
7. At the end of 48 hours, media was completely removed, cells were washed with PBS, and fixed using methanol.

13.3 Indirect immunofluorescent staining

The monolayer cells in 6 well plate was fixed with 3 ml of methanol per well for 30 min at room temperature. After decanting the methanol, the monolayer was washed thrice using PBS and 1:100 diluted monoclonal antibodies (R25 and R24, kindly gifted by Dr. Chinta Lamichhane) was added to the VP1 transfected well. Similarly, 1:100 diluted CAV positive polyclonal serum was added to the VP2 transfected well. The plate was incubated

at 4^0 C overnight. The wells were washed thrice with PBST and 1 ml of 1:30 diluted anti-mouse FITC (sigma, USA) was added to the VP1 transfected well and 1: 30 diluted anti-chicken FITC was added to the VP2 transfected well. The plate was incubated for 1 hour at room temperature. The wells were washed thrice with PBST and 1.5 ml of 50% (v/v) glycerol saline per well was added. The plate was visualized under a inverted UV microscope.

13.4 Bulk purification of the pVAX-CAV-VP1 and pVAX-CAV-VP2 plasmids

The recombinant plasmid was grown in 2 liters LB media for 18 hours in an orbital shaker cum incubator (Gallenkemp, USA). The bacteria were pelleted by centrifugation at 8000 rpm for 10 min. The pellet was lysed by adding the TENS (10ml for every 200 ml of bacterial culture) and votexing gently. Sodium acetate (3M, 5 ml) was also added to the above lysate and vortexed. The lysate was centrifuged at 12,000 rpm for 15 min. The fluid portion leaving the pellet was collected and precipitated using 2.5 volume of ethanol by keeping at -20^0C. The plasmid alongwith RNA was pelleted by centrifugation at 12,000 rpm for 30 min. The pellet was washed once with 70% ethanol, air-dried and dissolved in 3 ml of the TE buffer containing RNAse at a final concentration of 50 µg/ml and incubated for 1 hour in room temperature. It was extracted twice with phenol: chloroform (1:1) and once with chloroform, the plasmid in the acquous phase was precipitated using 2.5 volume of ethanol and 0.3 M concentration of sodium acetate at -20^0C. The pelleted plasmid was further purified by PEG precipitation as described by Sambrook *et al.* (1989).

13.5 Protective effect of CAV-VP1 and VP2 proteins

The protective effect of CAV proteins (VP1 and VP2) was studied using the nucleic acid immunization approach. The PEG purified expression plasmids pVAX-CAV-VP1 and pVAX-CAV-VP2 were used for this purpose. The study was carried out in 18 weeks old layer birds and in 18-day-old SPF embryos by *in ovo* vaccination approach.

13.5.1 Experimental study using the Layer birds

The experiment in layer birds was aimed to study the usefulness of CAV-VP1 and VP2 to prevent the vertical transmission of the virus. For this experiment, 30 birds were divided into three groups (1, 2 & 3) comprising of 10 birds in each group, which included 2 males and 8 females in each. Day prior to immunization, pre-immunization sera samples were collected from all the birds to check for the presence of CAV antibodies. Birds from the groups 1 & 2 received the plasmids at a dose rate of 200 µg (100 µg of pVAX-CAV-VP1 and 100 µg of pVAX-CAV-VP2) per bird. Birds in-group 2 received a booster dose of

plasmid (same dose) on 15th day from the first dose. Group 3 served as control for the experiment, which received the pVAX plasmid without any insert at the same dose rate. The birds in all the groups were monitored weekly 7, 14, 21 and 28 days post vaccination (DPV) for the cell-mediated immune response (CMI) by lymphocyte transformation assay and for humoral immune response (HIR) by ELISA. The fertile eggs were collected from 21 DPV and 28 DPV and incubated in the hatchery unit of Central Avian Research Institute (CARI), Izatnagar. The chicks hatched out were challenged at one day of age with 1 ml of cell culture fluid having $10^{4.5}$ $TCID_{50}$/0.1ml intramuscularly. The chicks were observed for clinical signs and mortality; body weight and PCV were taken at 5, 10 and 15 DPI. All the chicks were sacrificed on 15 DPI humanely and gross lesions were observed in different organs viz., thymus, bone marrow, spleen, liver and bursa. The number of birds showing lesions was recorded. Tissues of all these organs were collected in 10% formal saline for the histopathological studies and the number of birds showing microscopic lesions was recorded.

13.5.2 Lymphocyte Transformation Test (LTT)

The peripheral blood mononuclear cells were separated as per the method of Haddad and Mashly (1991). Equal volumes (3 ml) of blood samples from four chicks of each group, including control chicks, were separately drawn aseptically from jugular vein into sterile syringes containing EDTA (2 mg/ml) and collected in sterile tubes. Each 3 ml of blood was diluted separately with 2 ml of sterile PBS (pH 7.2) and was layered carefully over 2 ml of histopaque (1.077 g/ml, Sigma Chemical Co., USA) in screw capped 15 ml plastic centrifuge tubes. The tubes were centrifuged at 1500 rpm for 30 min. The interface layer ring rich in mononuclear cells was collected carefully and washed thrice with PBS by centrifugation at 1000 rpm for 10 min each time. Washed cells were collected in RPMI-Growth Medium. Viability of the cells was ascertained by trypan blue dye exclusion method and the cells were adjusted to give 5×10^6 viable cells/ml in RPMI-GM.

The MTT colorimetric assay for cellular proliferation was done following the method described by Bounous *et al.* (1992), with some necessary modifications. For non-specific stimulation, Con-A (10 µg/ml) was used, whereas for specific stimulation CAV propagated in MSB1 cells having an infectivity titre of $10^{4.5}$ $TCID_{50}$/ml at a 1: 10 dilution were used as antigen. Briefly, 100 µl of the above peripheral blood mononuclear cells suspension of each group was added to three sets of triplicate culture wells of 96-well, flat-bottomed tissue culture plates (Corning, USA). The first set of triplicate culture wells (control)

received 100 µl of RPMI-GM. The second and third sets of triplicate culture wells received 100 µl of RPMI-GM, containing 20 µg/ml of Con-A (final concentration 10µg/ml) and 100 µl of CAV antigen, respectively. The plates were incubated at 37°C in a humidified chamber at 5% CO_2 tension. After 72 hr, 20 µl of MTT dye solution (5 mg/ml) was added to all the wells and incubated further for 4 hr, following which the plate was centrifuged at 1200 rpm for 15 min. The supernatant was removed carefully from all the wells and then 150 µl of DMSO was added in each well for the extraction of MTT formazan from the cells. The contents were mixed properly and after 15 minutes, the OD of wells was measured at a test wavelength of 510 nm and a reference wavelength of 650 nm. Stimulation index (SI) was calculated by dividing the OD value of stimulated with the OD of unstimulated.

13.5.3 Detection of CAV antibodies by ELISA

The antibody against CAV was detected by employing the CAV antibody detection kit as described by the manufacturer. (Synbiotics, USA. Kindly gifted by Dr. Chinta Lamichinne). The titre was calculated as described by the manufacturers equation. Briefly, the optical density of average positive serum and average normal control serum was calculated from the three wells of each plate. The corrected positive control value was calculated by subtracting the average normal control value from the average positive control value. The sample to positive ratio was calculated by subtracting the average normal serum from each sample absorbance and the difference was divided by the corrected positive control. The equation is given below.

$$SP = \frac{\text{(Sample absorbance) - (Average normal control serum)}}{\text{Corrected positive control absorbance}}$$

The ELISA tire was calculated using the following calculation as suggested by the manufacturer.

Log_{10} Titre =(1.009 X Log_{10} SP) + 3.628

Titre = Antilog of log 10 Titre.

13.6 *In ovo* vaccination

In ovo vaccination was carried out in 18-day-old SPF embryos as described by Kapczynski *et al.* (2003). Forty embryos were divided into two groups containing 20 embryos in each. Embryos in the group 1 received 100 µg of expression plasmids (50 µg of pVAX-CAV-VP1 and 50 µg of pVAX-CAV-VP2) per embryo. The chicks from the two groups were hatched out separately and reared in strict isolated

conditions. After hatching, the groups were made to 15 in each. The sera samples were collected on 7, 14, 21 and 28 days post hatching to monitor the antibody development. The chicks were challenged on 15th day with the chicken anemia virus at a dose of 1 ml ($10^{4.5}TCID_{50}$/ 0.1ml) per bird intramuscularly. The chicks were observed for the clinical symptoms and mortality daily. The PCV and body weight was taken in 5,10 and 15 days post infection. At 15th day post challenge, all the chicks were sacrificed humanely and gross lesions were observed in different organs viz., thymus, bone marrow, spleen, liver and bursa. The number of birds showing lesions was recorded. All these tissues were also collected in 10% formal saline for the histopathological studies and the number of birds showing microscopic lesions was recorded.

14. ANTINEOPLASTIC EFFECT OF THE CAV-VP3 PROTEIN

14.1 PCR amplification of the VP3 gene

The PCR amplification of the CAV-VP3 gene was carried out using the primer set #4 (VP3F & VP3R). The reaction was carried out with an initial denaturation of 3 min followed by 35 cycles of denaturation at 94^{0}C for 1 min, annealing at 58^{0}C for 1 min, and extension at 72^{0}C for 1 min, and the final extension carried out at 72^{0}C for 5 min. The specific PCR amplified product of 367 bp was purified from the gel and used for cloning into the expression vector.

14.2 Cloning of the VP3 gene

The CAV VP3 gene was cloned in the pVAX similarly as described for the VP1 and VP2 in section 13.1.

14.3 Analysis of *in vitro* expression

The plasmid for *in vitro* expression study was purified using the plasmid Midi prep kit (Qiagen, Germany). *In vitro* expression of pVAX-CAV-VP3 was analyzed in the Hep 2 cells by indirect immunofluorescent assay as described earlier in section 13.3. For the fluorescent staining, CAV positive polyclonal sera and anti-chicken FITC conjugate were used.

14.4 Bulk purification of the pVAX-CAV-VP3

The expression plasmid pVAX-CAV-VP3 was bulk purified using the Mega/Giga plasmid purification kit as described in the manufacturers protocol (Qiagen, Germany).

14.5 *In vitro* study of anti-neoplastic effect of VP3

VP3 expression plasmid PVAX-CAV-VP3 was transfected in RSV transformed CEF cells and cytomorphological alterations were studied by acridine orange- ethidium bromide staining and indirect immunofluorescence technique.

Chicken embryo fibroblast (CEF) cell cultures were prepared from 10-11 day-old chicken embryos as per the method described by Merchant *et al.* (1960) with slight modifications. The eggshells were thoroughly cleaned with cotton wool, moistened with 70% alcohol and cut open at previously marked air space. With the help of sterile forceps, the embryos were removed out and then washed thrice with Hank's balanced salt solution (HBSS). Using sterile scissors, the head and appendages of the embryos were separated from the body and the abdominal wall was cut open to remove the visceral organs. The tissues were then washed with sterile HBSS and were finally minced into very small pieces. The minced tissues were suspended in ten times the volume of 0.25% trypsin solution (kept warm at 37°C), added to trypsinization flask and stirred on a magnetic stirrer for 15 min at room temperature. The cell suspension was then filtered through a gauge filter and cells in the filtrate was pelleted by centrifuging at 2000 rpm for 10 min and then washed twice in HBSS and once in growth medium (Glasgow Modified Eagle's Medium with 10% serum). The cell concentration was adjusted to 2 x 10^6 cells per ml of growth medium (approx. 1:200). The freeze-dried Rous sarcoma virus was dissolved in 1ml of HBSS and filtered in 0.4-micron syringe filter. The filtrate was mixed with CEF cells and seeded in 6 well culture plate (2 ml per well) and incubated in a CO_2 incubator. After 24 hr, the monolayer cells were transfected with CAV VP3 recombinant plasmid pVAX-CAV-VP3 and apoptotic changes were studied by indirect immunofluorescence technique and acridine orange- ethidium bromide staining.

14.6 *In vivo* study of anti-neoplastic effect of VP3

SPF chicks were divided into four groups with 10 chicks in each were used for studying the anti-neoplastic effect of CAV VP3. The experiment was carried out as per the following schedule.

Group 1 - RSV inoculated on day 1 and pVAX plasmid was injected intra-tumoraly on day 10.

Group2 - RSV inoculated on day 1 and pVAX-CAV-VP3 plasmid was injected intra-tumoraly on day 10.

Group 3 - pVAX plasmid was injected intramuscularly on day 1 and RSV was inoculated on day 10.

Group 4 - pVAX-cAV-VP3 plasmid was injected intramuscularly on day 1 and RSV was inoculated on day 10.

In group 1 and 2, all the birds were sacrificed at 20th day of age to study the bodyweight: tumor ratio and tumor tissues were collected. The collected tissues were analyzed for the cytomorphological changes of apoptosis.

Acridine orange - Ethidium bromide staining: Fine cryosections of 2-3 µm of tumor tissues were cut in the cryotome (IEC, UK). The sections were fixed in clean microscopic slides by keeping in chilled acetone for 15 min. The fixed slides were stained as described below.

Fluorescent Antibody Technique: The sections made as described earlier were washed thrice in PBS and 1:50 a diluted anti-CAV chicken serum was added. The slide was incubated for 1 hour at 37^0 C in a moist chamber. Then, it was washed thrice with PBST and 1:30 diluted anti-chicken FITC conjugate was added and incubated for 1 hour at 37^0 C. After washing thrice, the slide was air-dried and 20 µl of 50% glycerol saline was added. Coverslip was placed in a glass slide and visualized under the UV microscope (Nikon, Japan).

Histopathology: The tumor tissues were collected in 10% formalin and fine sections made in microtome. The sections were stained using hematoxylin and eosin stains and studied for the apoptotic changes under microscope.

1. VIRUS PROPAGATION AND CONFIRMATION

All the CAV field isolates were adapted in MDCC-MSB-1 cells. The cytopathic changes of cellular degeneration were observed after 5 - 6 subculturing of the infected cells. The virus growth in MSB-1 cells was further confirmed by CAV-specific PCR using the DNA extracted from the infected MSB-1 cells (Fig. 3) as reported by Dhama *et al.* (2002).

2. MOLECULAR CHARACTERIZATION OF CAV

2.1 PCR amplification of different regions of CAV genome

The primer sets of #1, #2 and #3 were used for the amplification of VP1, VP2 and non-coding regulatory sequence of CAV genome, respectively. PCR amplification using primer set #1 yielded a specific product of 1390 bp (Fig. 4). PCR amplification using primer set #2 and #3 yielded specific products of expected size i.e. 713 and 572 bp (Fig. 5 and Fig. 6), respectively. The PCR amplified products were confirmed by their expected size in agarose gel, RE analysis and nucleotide sequencing.

2.2 Restriction endonuclease analysis

The purified VP2 products of isolate A, B, C, E and P were digested with *Hae* III and *Mbo* I enzymes. Analysis of the amplified VP2 region of different isolates using the restriction enzyme *Hae* III revealed that CAV-E was different from other isolates (Fig. 7) whereas *Mbo* I enzyme did not reveal any difference among the CAV isolates (Fig. 8).

Restriction profile study of PCR amplified VP1 region of different isolates revealed differences among the Indian isolates. *Hha* I enzyme differentiated CAV-A, B or P and E isolates by yielding different pattern (Fig. 9). With *Dde* I enzyme the isolates could be assigned two groups in which A and E in one and B and P in another (Fig. 10), whereas *Sac* I digestion grouped A and P isolates together, and B and E together in two groups separately (Fig. 11). *Hae* III digestion of the PCR amplified VP1 differentiated CAV-E from CAV-A, B and P isolates (Fig. 12). Using these three enzymes all the Indian isolates could be differentiated.

2.3 Sequence alignment

A nucleotide sequence portion of 1766 bp containing the complete coding region of VP2 (which also included the VP3) and partial coding region of VP1 protein of four Indian isolates A, B, E and P were aligned in the MegAlign programme of Lasergene Software

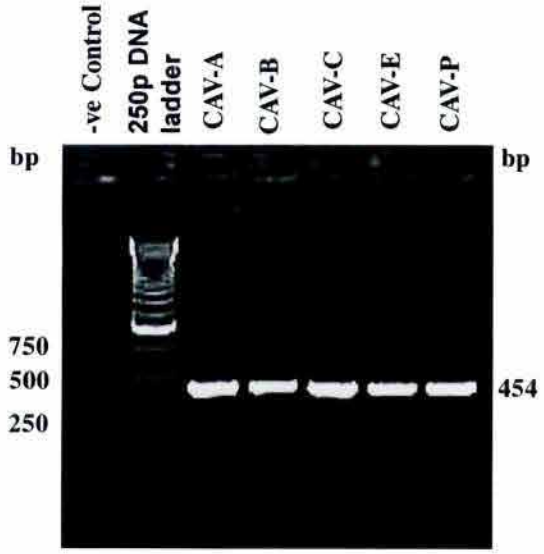


Fig. 3: Confirmation of CAV isolates by CAV specific PCR

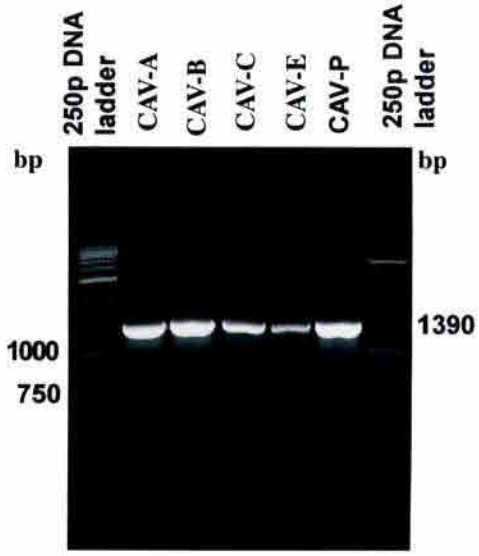


Fig. 4: PCR amplification of VP1 region of 5 Indian CAV isolates

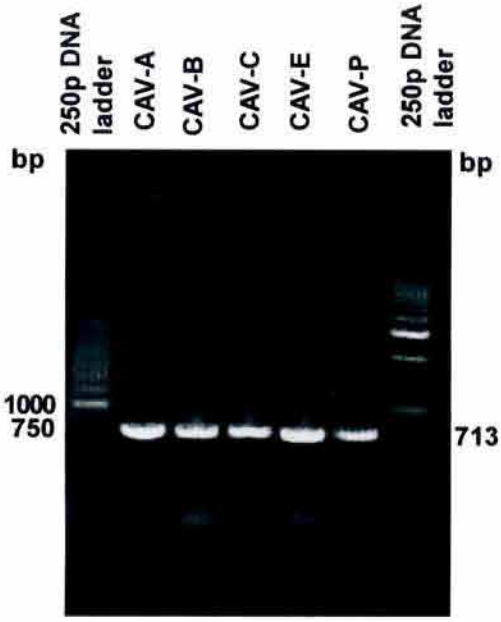


Fig. 5: PCR amplification of VP2 region of 5 Indian CAV isolates

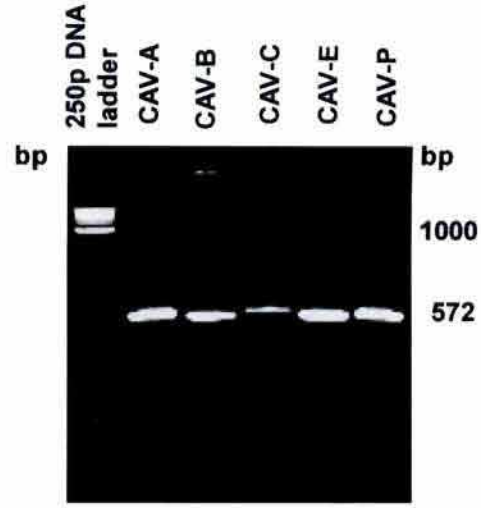


Fig. 6: PCR amplification of non-coding region of 5 Indian CAV isolates

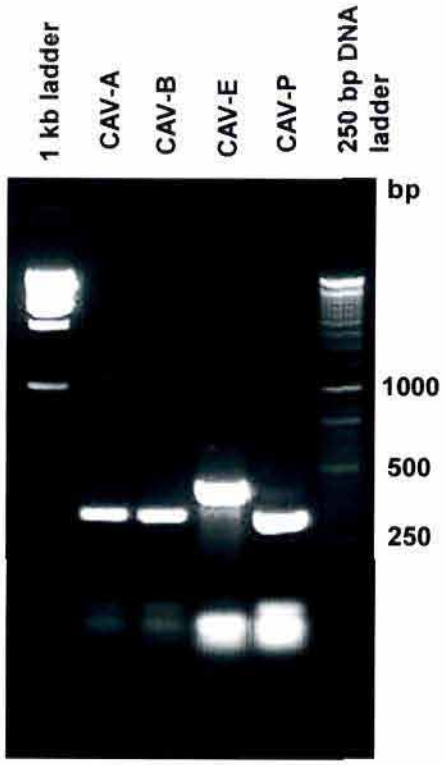


Fig. 7: Analysis of PCR amplified VP2 region of Indian CAV isolates using restriction enzyme *Hae* III

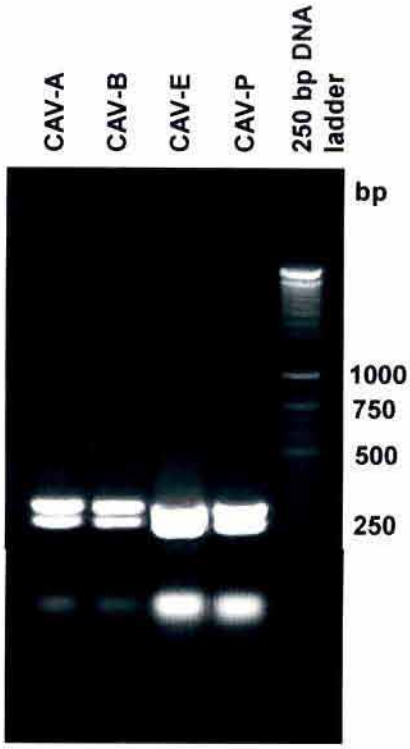


Fig. 8: Analysis of PCR amplified VP2 region of Indian CAV isolates using restriction enzyme *Mbo* I

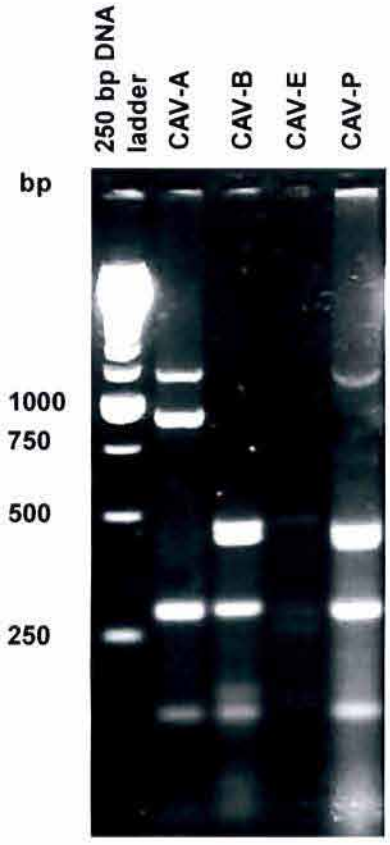


Fig. 9: Analysis of PCR amplified VP1 region of Indian CAV isolates using restriction enzyme *Hha* I

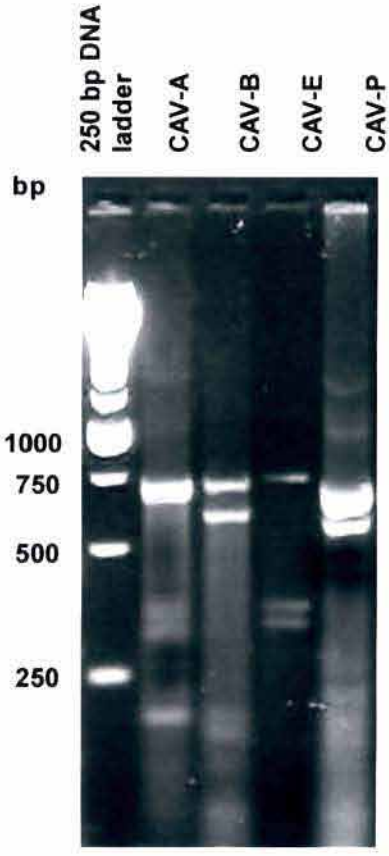


Fig. 10: Analysis of PCR amplified VP1 region of Indian CAV isolates using restriction enzyme *Dde* I

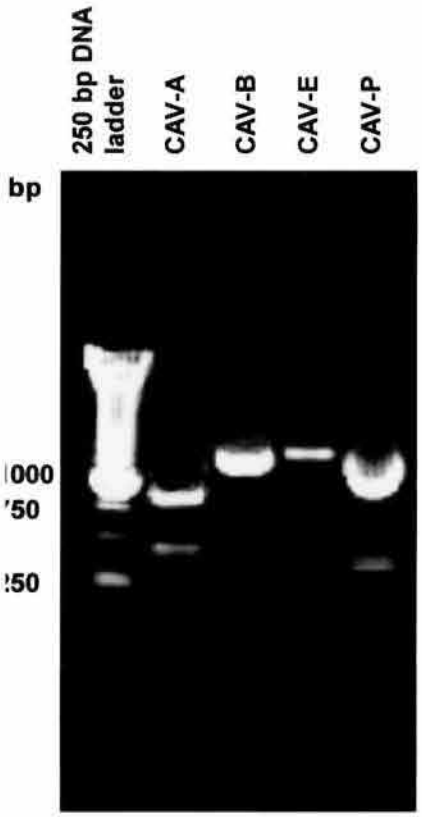


Fig. 11: Analysis of PCR amplified VP1 region of Indian CAV isolates using restriction enzyme *Sac* I

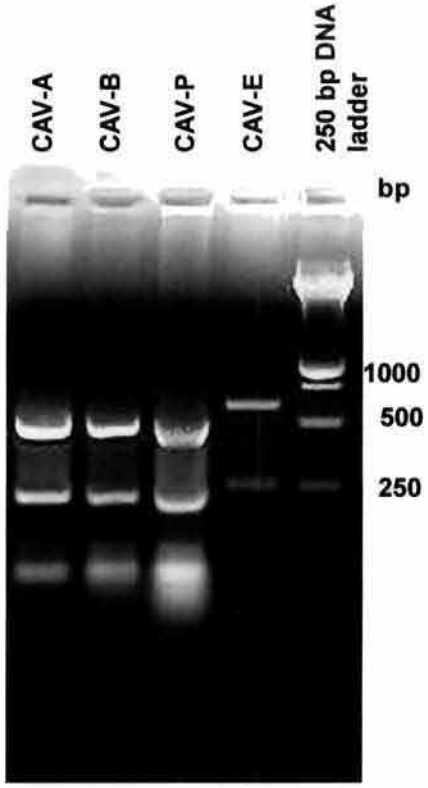


Fig. 12: Analysis of PCR amplified VP1 region of Indian CAV isolates using restriction enzyme *Hae* III

(DNA STAR, USA). The alignment report is presented in Fig.13. The alignment of deduced partial 431 amino acid sequence of VP1 of four CAV isolates (A, B, E and P) is also given in Fig. 14. Similarly the deduced VP2 amino acids sequences of five Indian CAV isolates (A, B, C, E and P) were aligned with the sequence of foreign isolates and alignment report is given in Fig. 15.

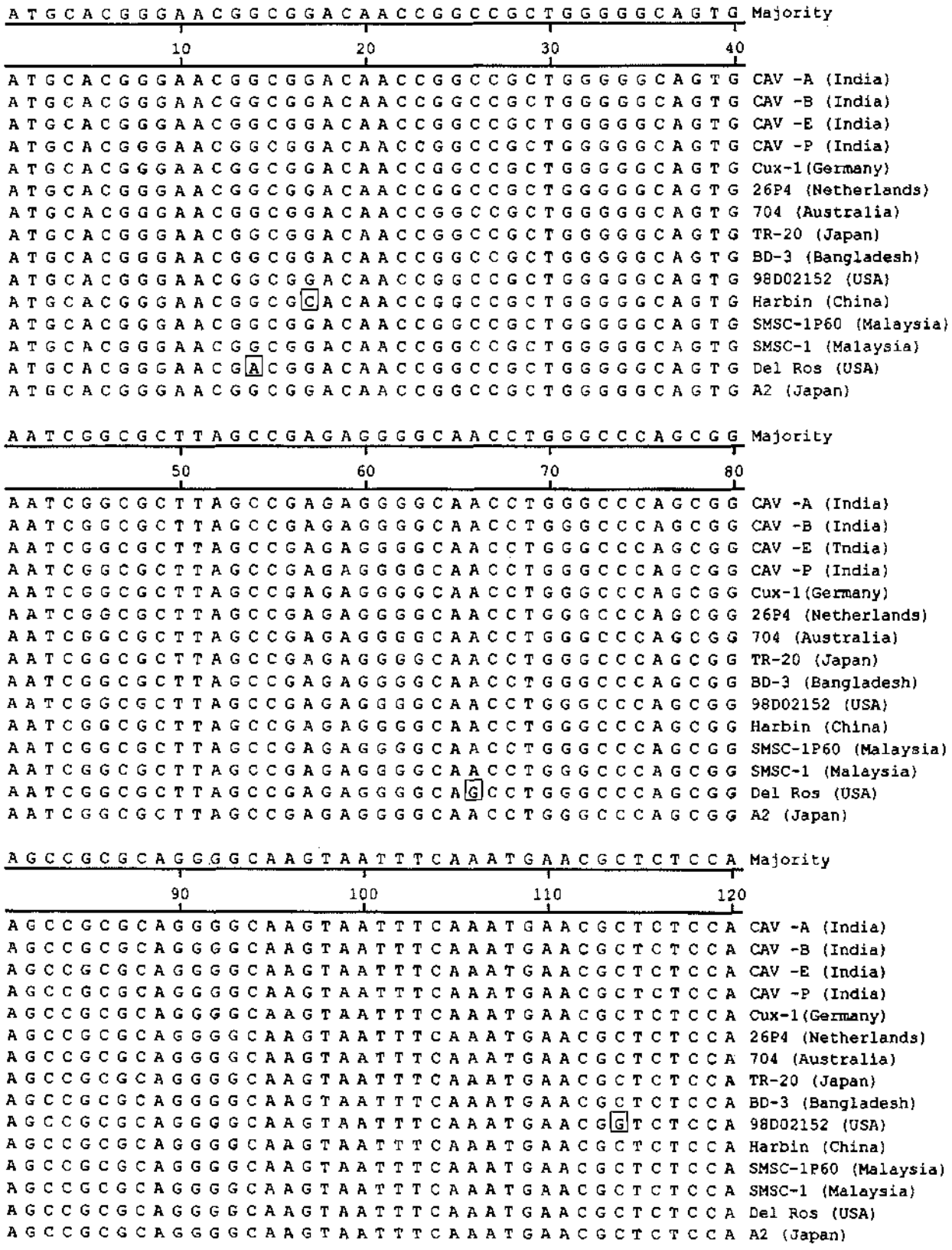
2.4 Sequence analysis

Analysis of the nucleotide sequence of 1766 bp from position 386 to 2151 (as per Meehan *et al.*, 1992) revealed 12 to 52 nucleotides variation in Indian isolates. A maximum variation of 52 nucleotides was observed in CAV-E isolate followed by 15 nucleotides in CAV-P, 13 in CAV-A and 12 nucleotides in CAV-B. Though 52 nucleotide changes in CAV-E were observed but it resulted in only 5 amino acids changes. CAV-E showed a maximum variation of 4% among the Indian isolates, however, the total variation ranged from 0.5 to 4.0% The CAV-E isolate had a maximum identity with the Australian 704 (99.3%) followed by Japanese strain TR-20 (99.2%) and Malaysian SMSC-1 (99%). All the Indian and foreign CAV isolates used for analysis showed overall nucleotide variation of 0.2 to 4.1% (Table 4). The Indian isolate CAV-B and CAV-P had maximum identity of ≥ 99% with Bangladesh isolate BD-3 whereas isolate CAV-A was very close to the European Cux-1 strain.

The partial coding nucleotide sequence of the VP1 region of CAV was translated into amino acid sequence using EditSeq programme of the Lasergene Software. The deduced partial 431 amino acids sequence alignment of VP1 revealed the occurrence of maximum variation of 7 amino acids in CAV-P, of which at positions 144 (H), 262 (D) and 275 (F) were found specific to the isolate. The other isolates CAV-A, B and E showed only 5, 6 and 5 amino acids variation, respectively. The amino acid variation at 144 (D) was specific for CAV-A isolate. No specific variation of amino acid unique to the isolate was observed in CAV-B. Amino acid identity based on the partial VP1 sequence (431 aa) indicated that the overall variation ranged from 0 to 2.8% (Table. 5). Among the Indian isolates, the variation ranged from 0.7 to 2.1%. CAV-E showed 100% identity with the Australian 704 strain. Similarly, CAV-B showed 100% identity to Bangladesh BD-3 strain while CAV-A had 99.5% identity to the European Cux-1 strain.

Analysis of deduced complete 216 amino acids sequence of VP2 protein of the Indian isolates revealed only one amino acid variation at position 149 in CAV-A and one at position 186 in CAV-E. Amino acid alignment of the VP2 (216 aa) region showed variation

Fig. 13. Alignment of nucleotide sequences of 1766 bp coding region of different chicken anaemia viruses. Nucleotides differing from majority are boxed.


ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG Majority
10
20
30
40
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG CAV -A (India)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG CAV -B (India)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG CAV -E (India)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG CAV -P (India)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG Cux-1(Germany)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG 26P4 (Netherlands)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG 704 (Australia)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG TR-20 (Japan)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG BD-3 (Bangladesh)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG 98D02152 (USA)
1 ATGCACGGGAACGGCGCACAACCGGCCGCTGGGGGCAGTG Harbin (China)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG SMSC-1P60 (Malaysia)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG SMSC-1 (Malaysia)
1 ATGCACGGGAACGACGGACAACCGGCCGCTGGGGGCAGTG Del Ros (USA)
1 ATGCACGGGAACGGCGGACAACCGGCCGCTGGGGGCAGTG A2 (Japan)
AATCGGCGCTTAGCCGAGAGGGGCAACCTGGGCCCAGCGG Majority
50
60
70
80
41 AATCGGCGCTTAGCCGAGAGGGGCAACCTGGGCCCAGCGG CAV -A (India)
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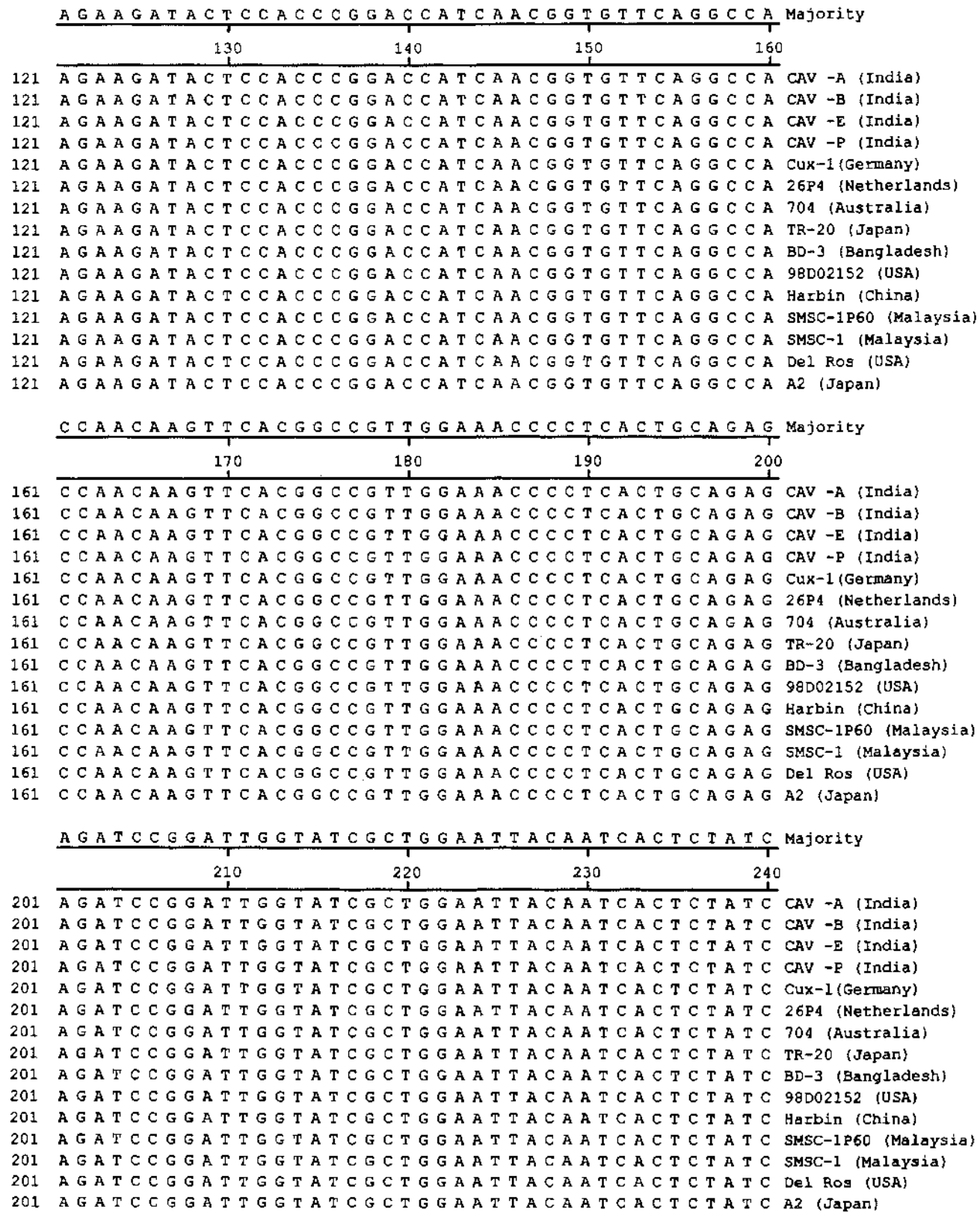
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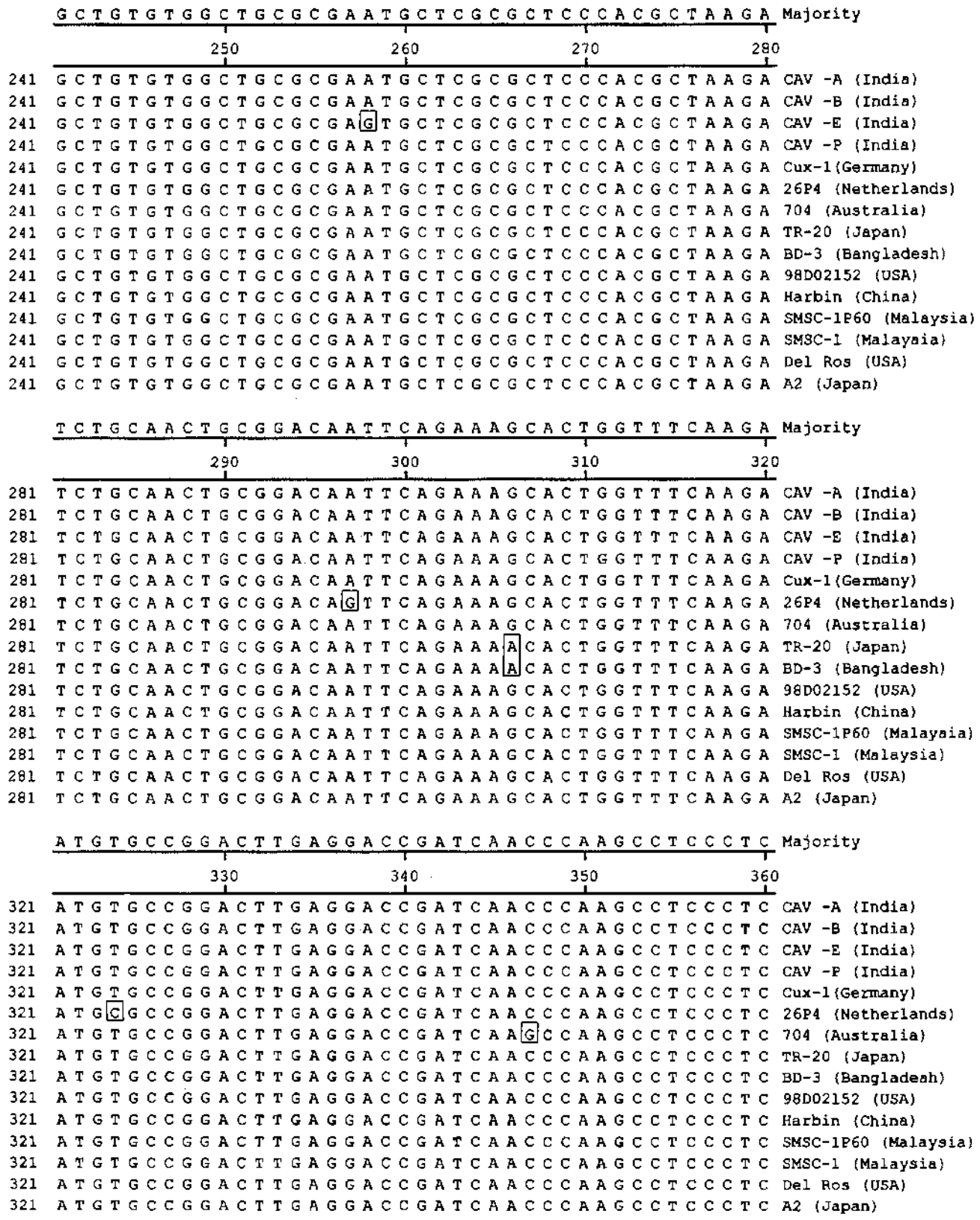
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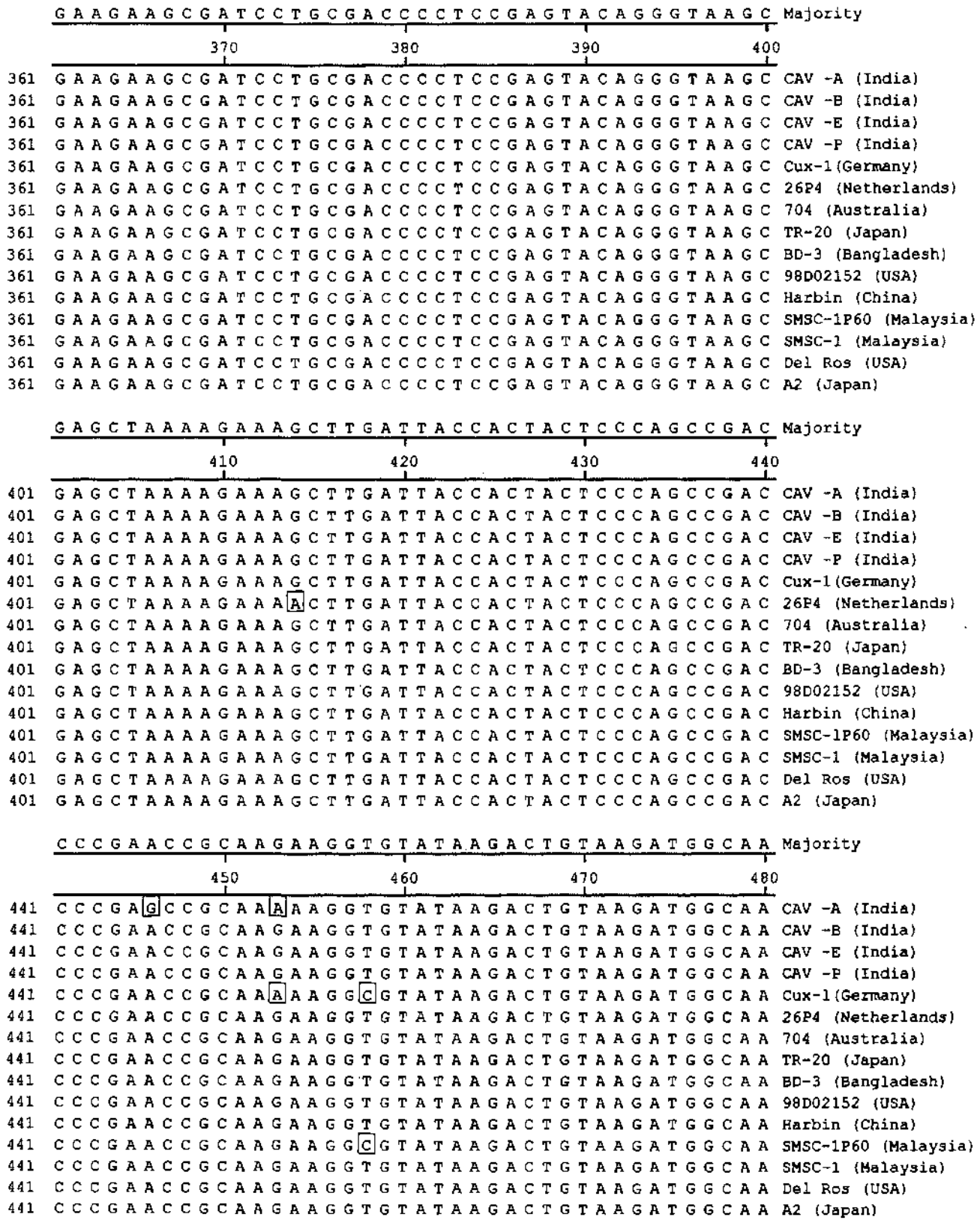
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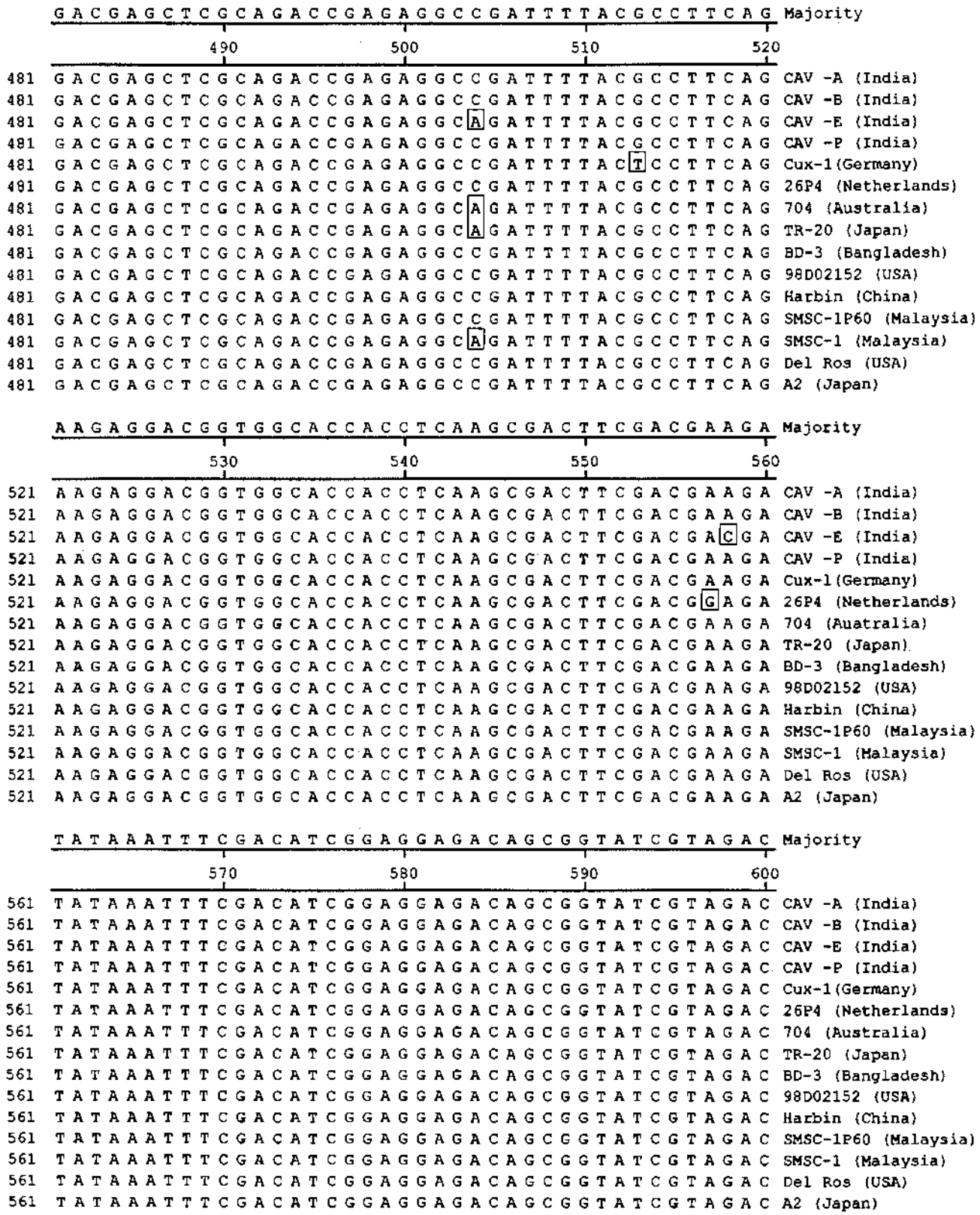
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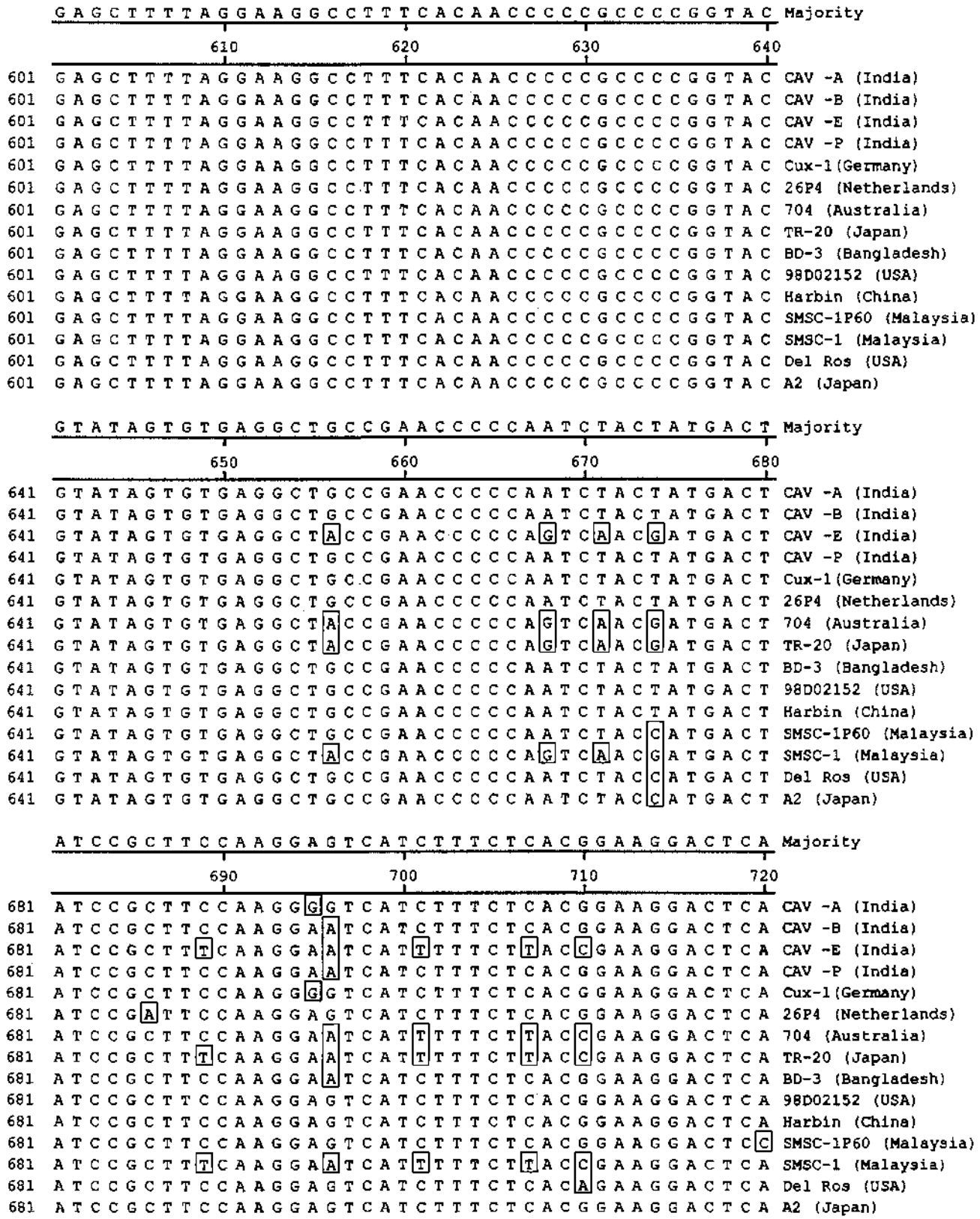
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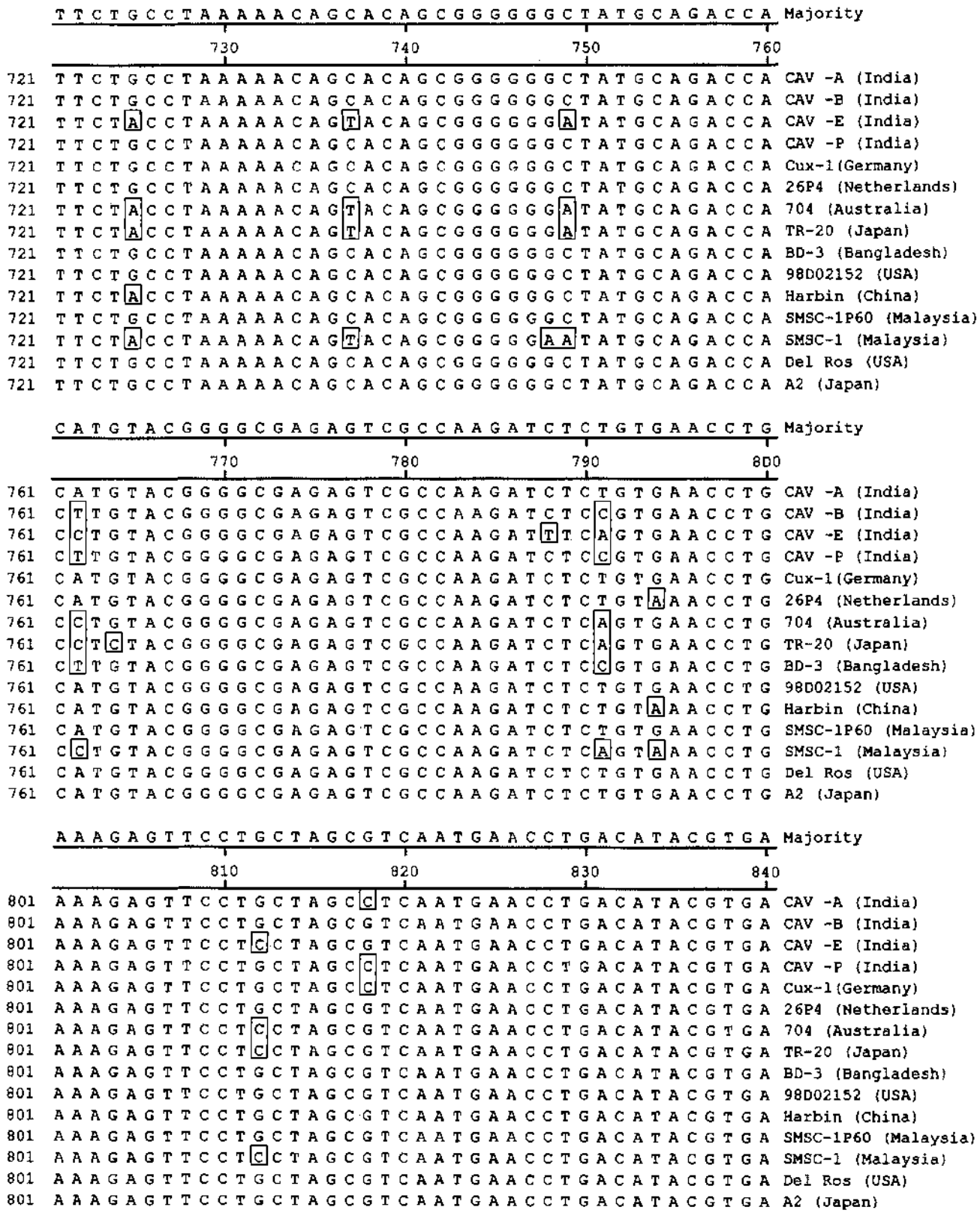
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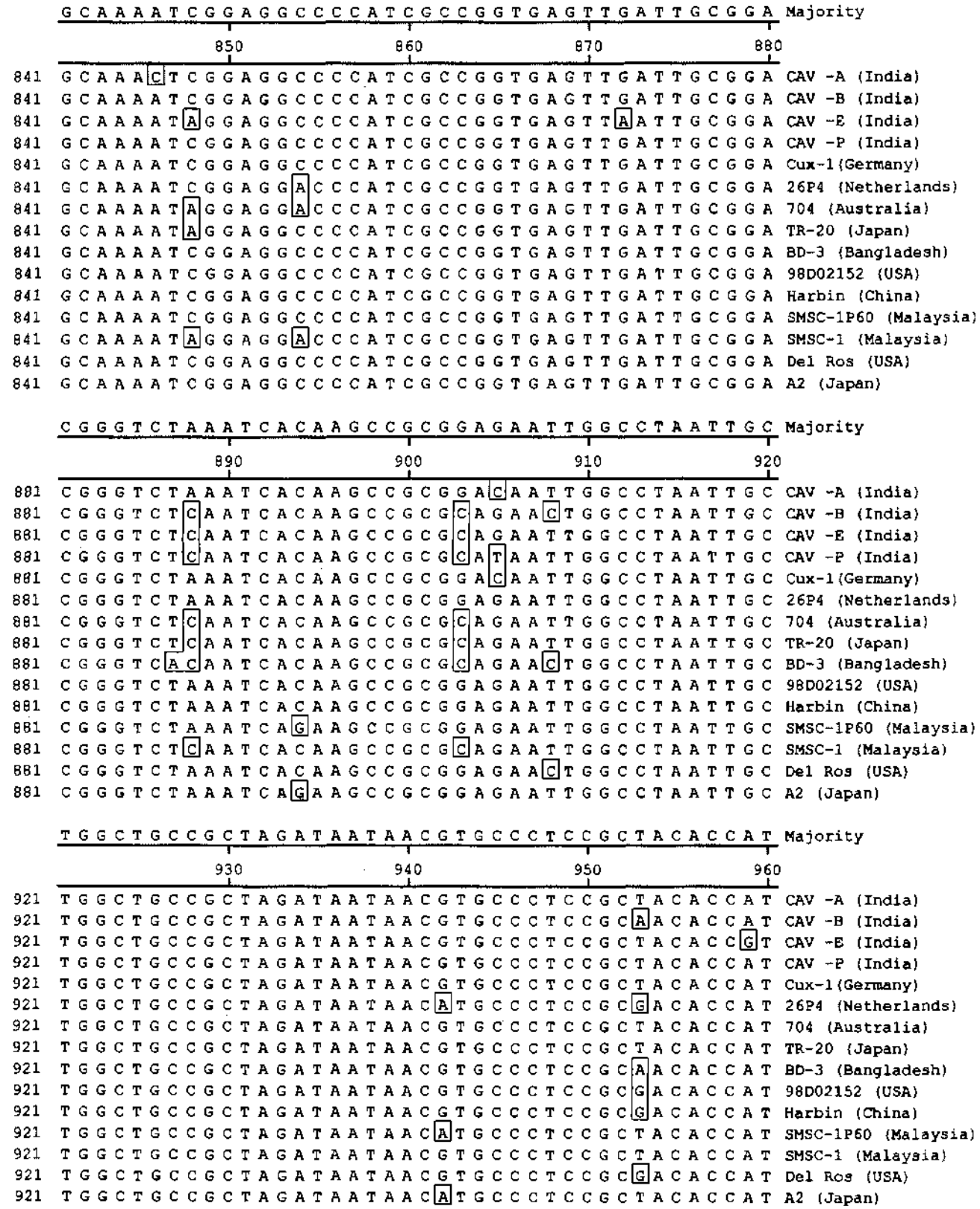
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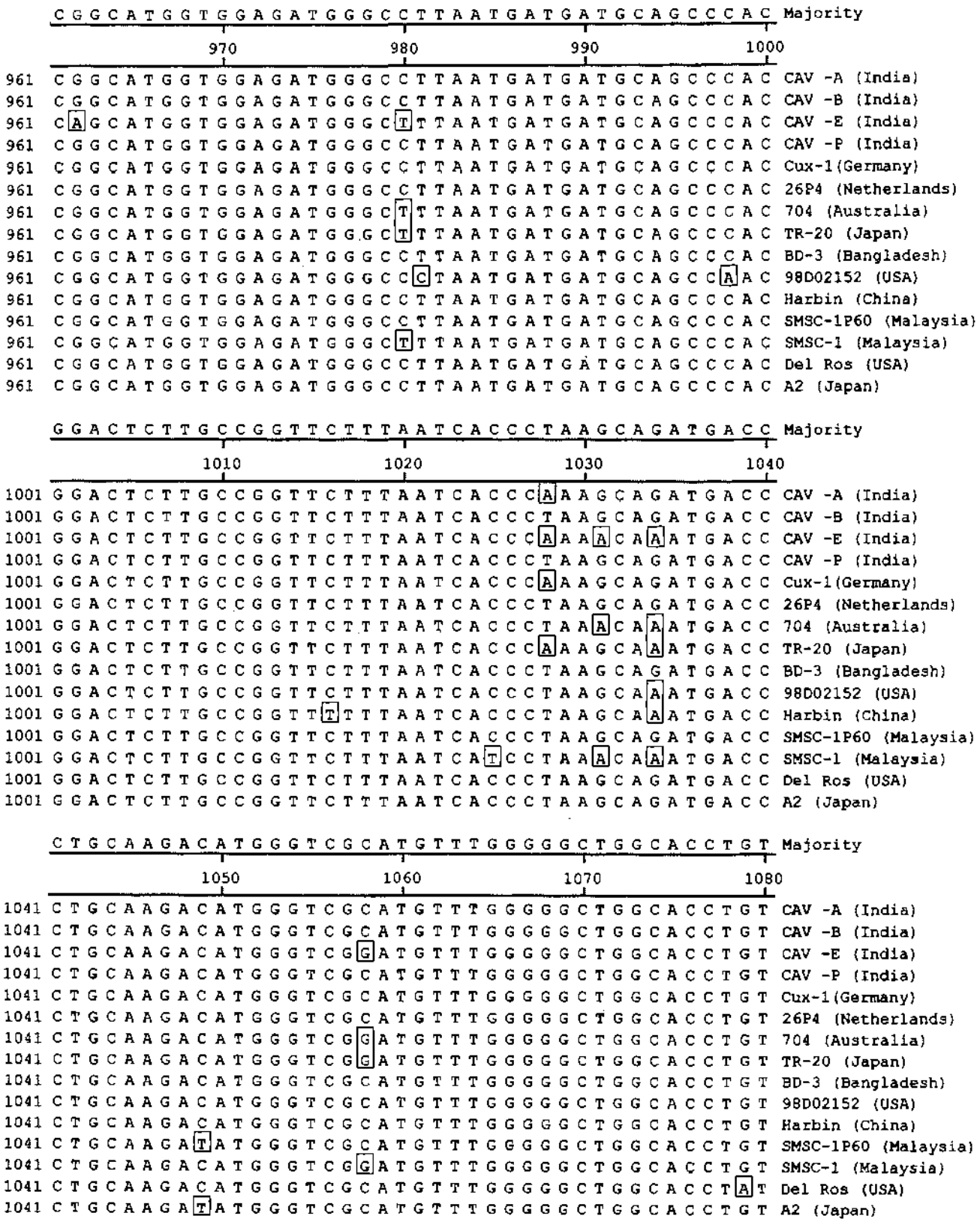
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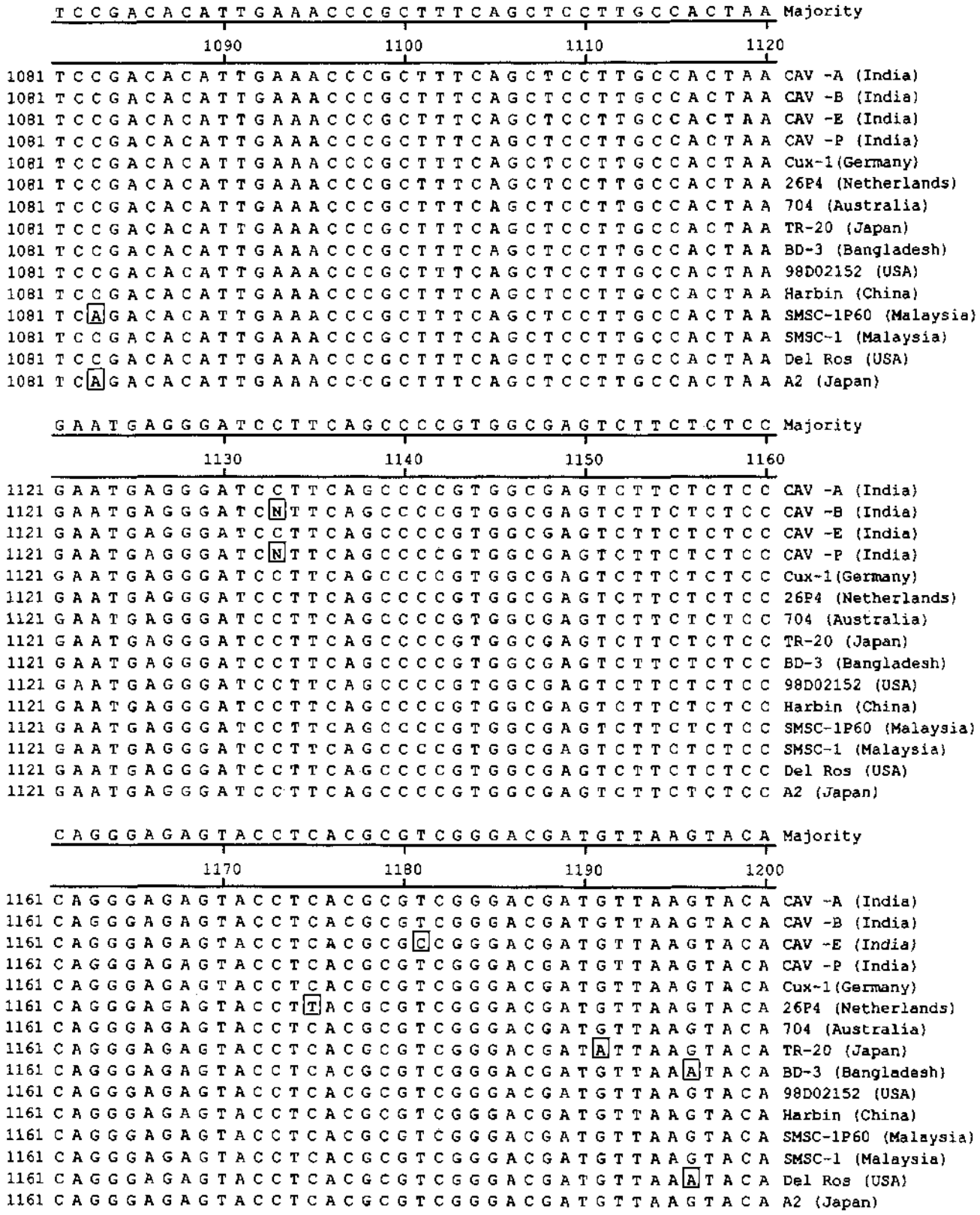
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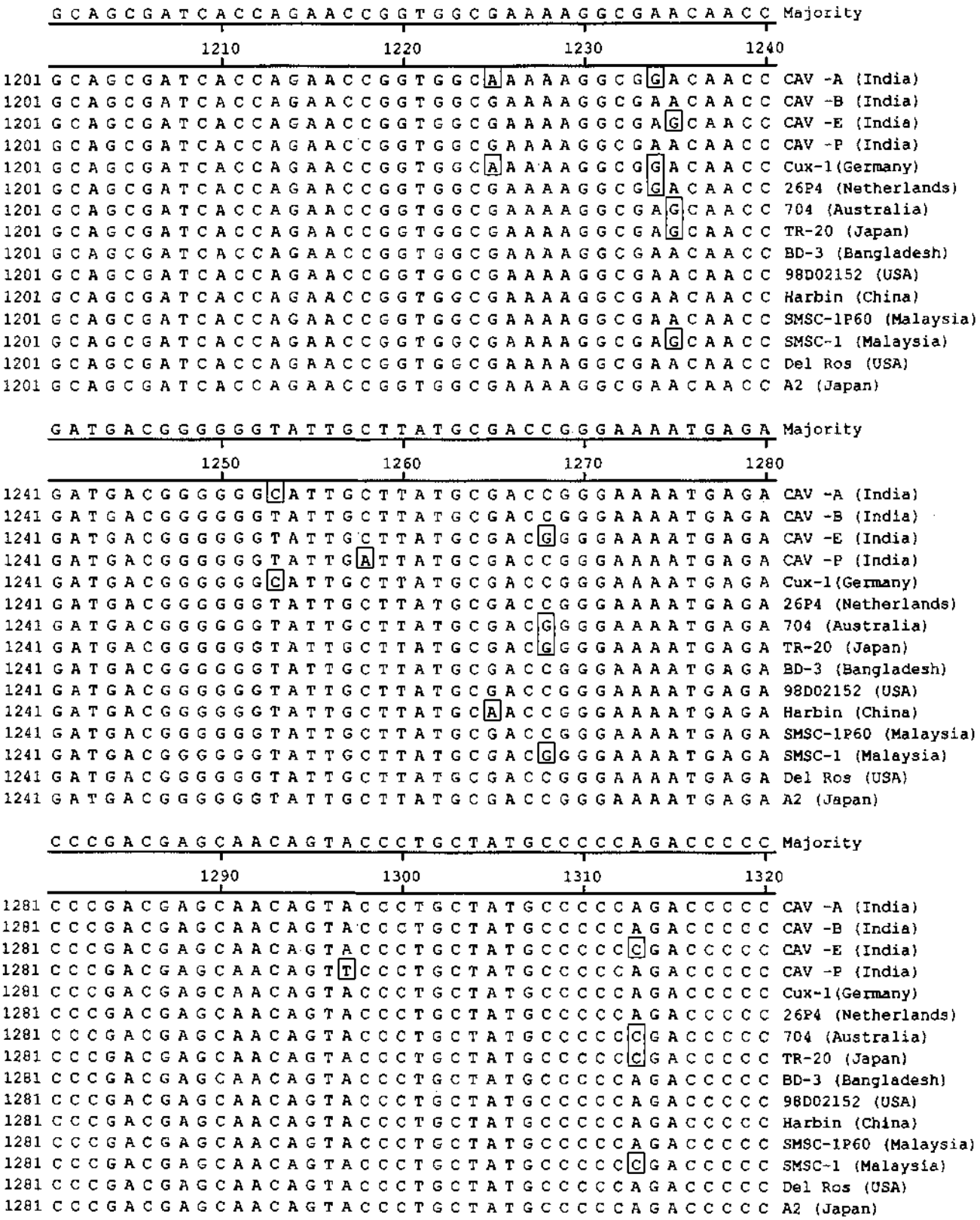
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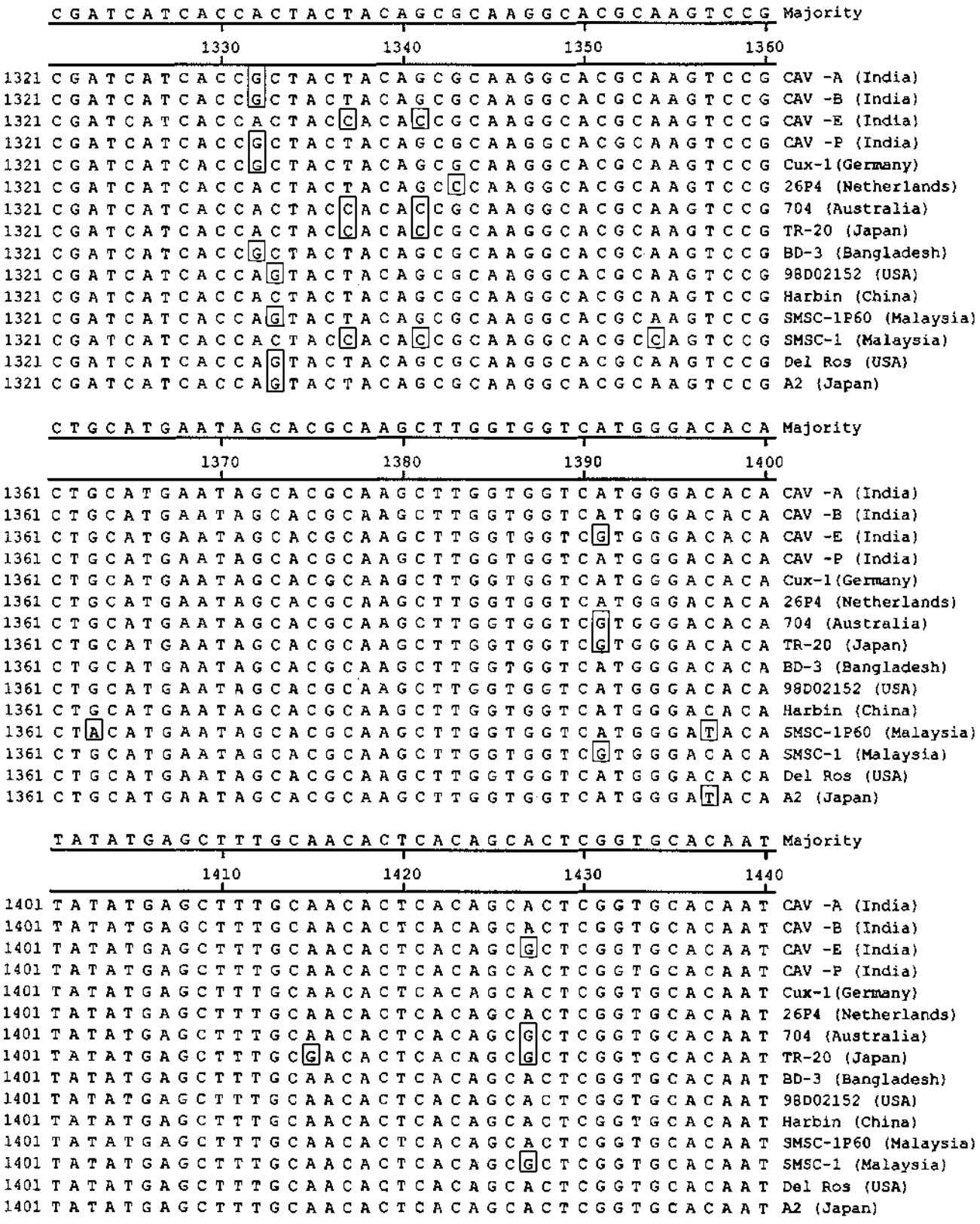
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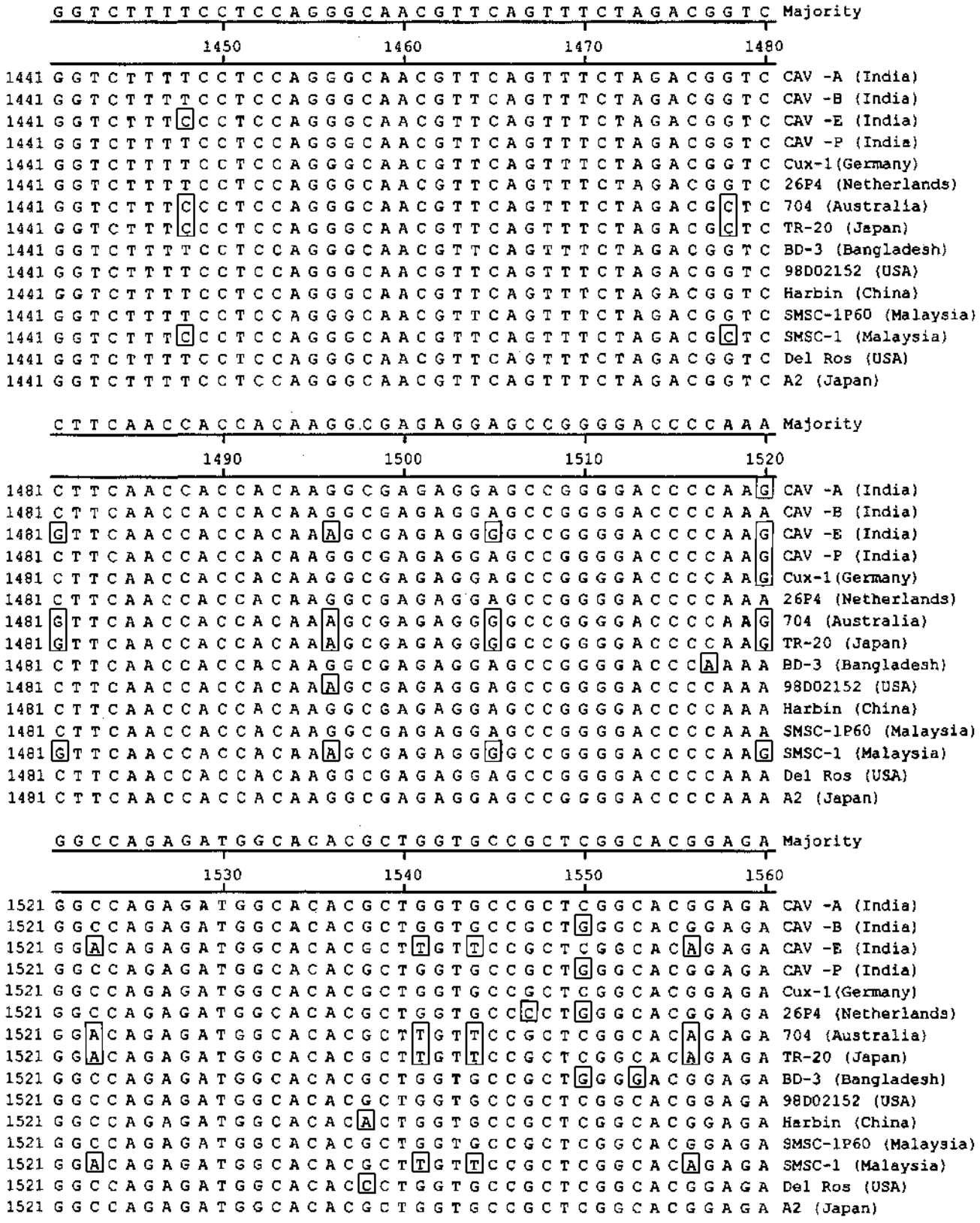
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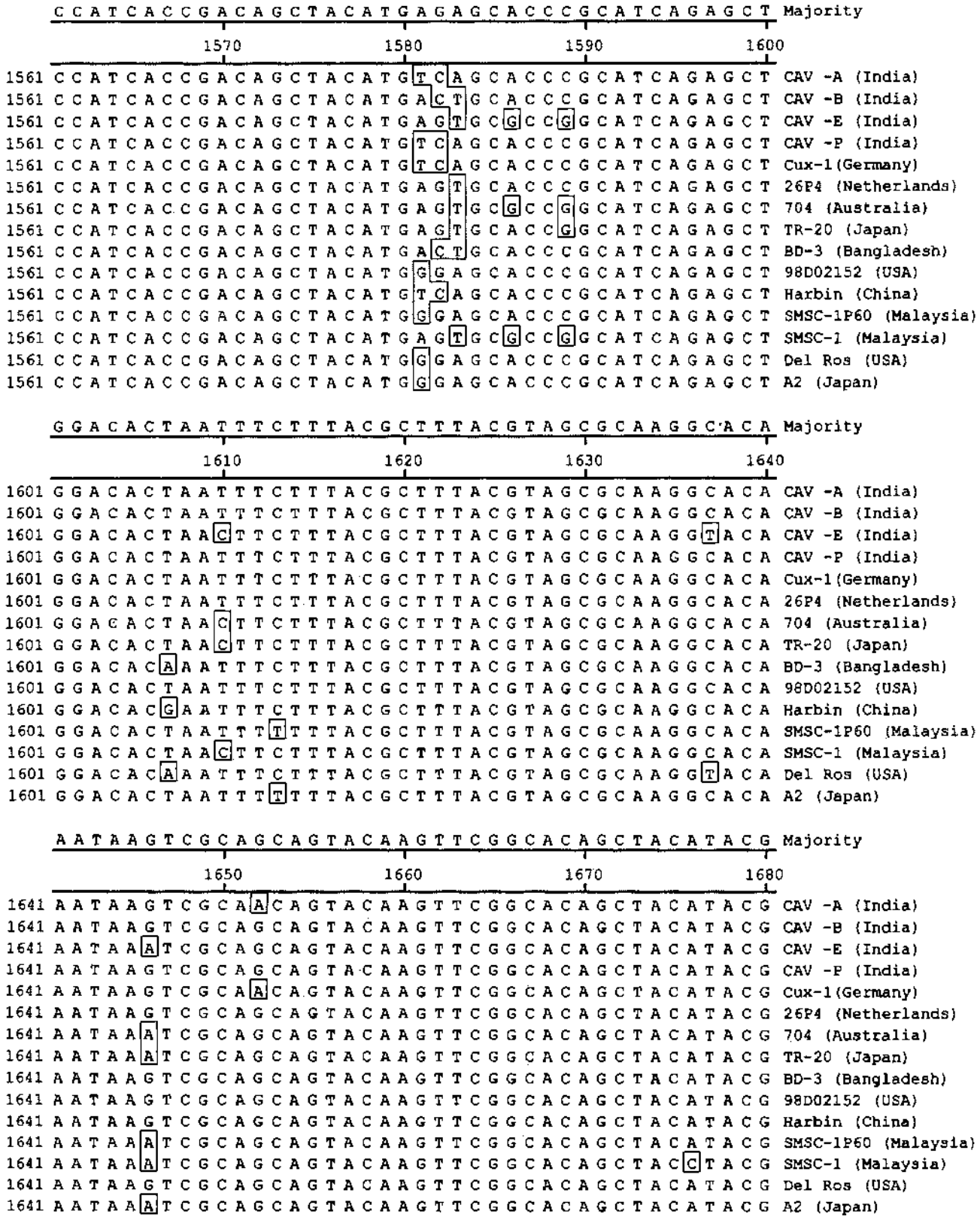
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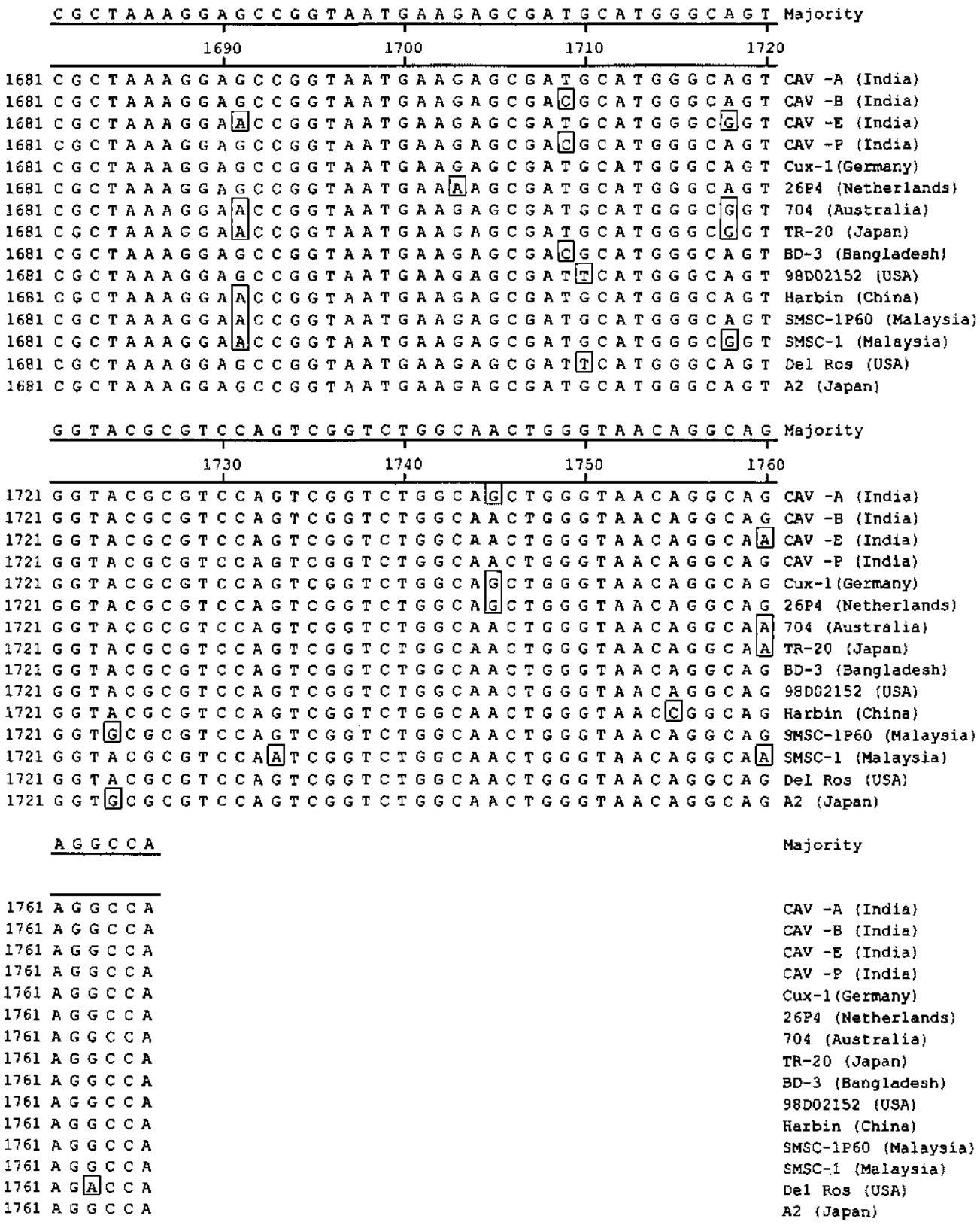


Decoration 'Decoration #1': Box residues that differ from the Consensus.

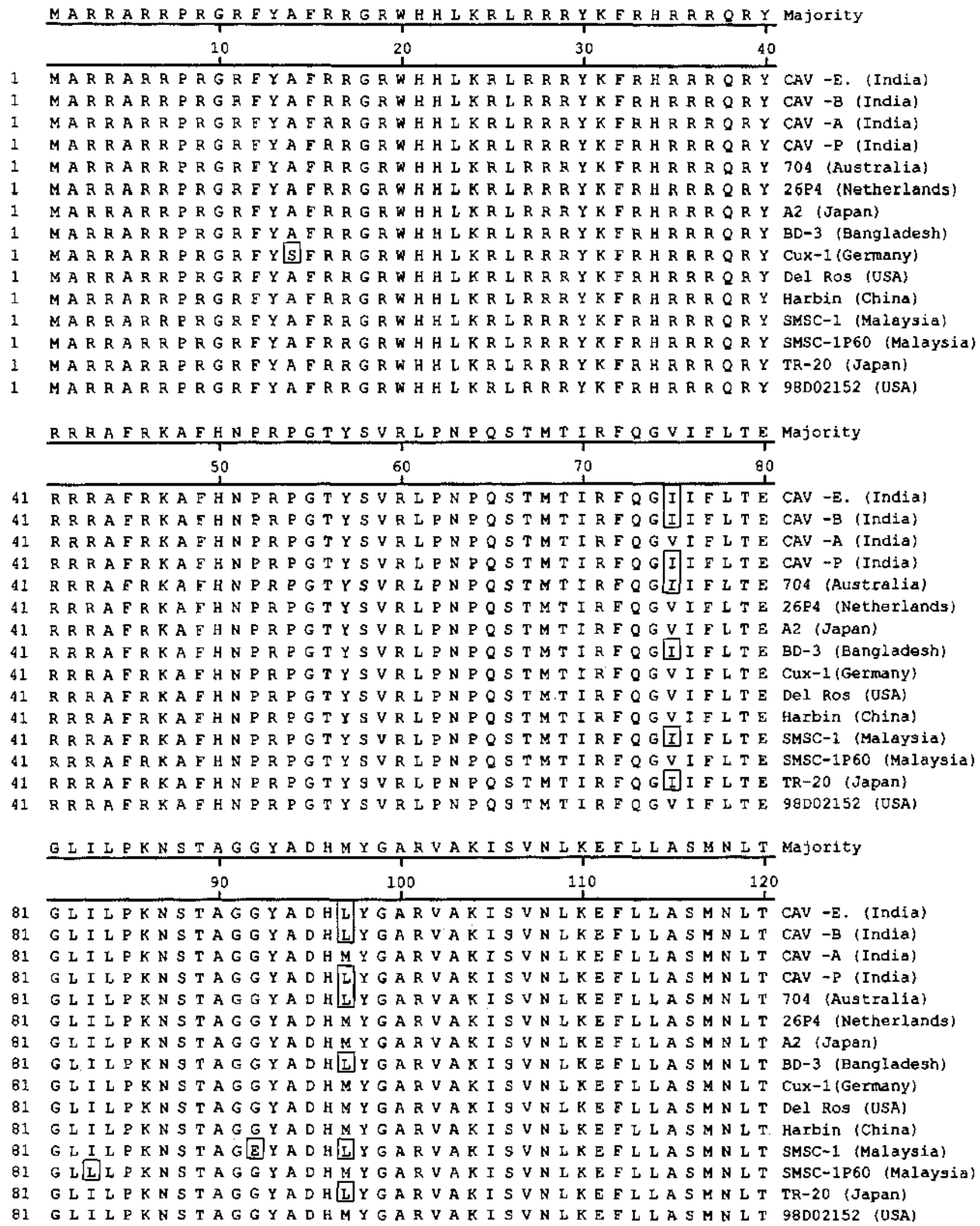
Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		98.5	96.0	98.8	99.8	98.4	96.3	96.3	98.2	98.6	98.6	98.3	96.0	98.3	98.5	**1**	CAV -A (India)
2	1.4		96.7	99.4	98.5	98.6	97.1	96.9	99.6	98.8	98.6	98.4	96.7	98.6	98.6	**2**	CAV -B (India)
3	4.0	3.4		96.5	96.1	95.9	99.3	99.2	96.4	96.5	96.4	96.2	99.0	96.1	96.3	**3**	CAV -E (India)
4	1.1	0.5	3.5		98.8	98.2	96.9	96.7	99.0	98.6	98.5	98.2	96.5	98.2	98.5	**4**	CAV -P (India)
5	0.2	1.4	4.0	1.1		98.4	96.4	96.3	98.2	98.6	98.6	98.4	96.0	98.3	98.5	**5**	Cux-1(Germany)
6	1.7	1.4	4.1	1.7	1.7		96.4	96.1	98.3	98.6	98.5	98.2	96.2	98.2	98.5	**6**	26P4 (Netherlands)
7	3.7	2.9	0.7	3.1	3.7	3.6		99.4	96.8	96.9	96.8	96.6	99.5	96.4	96.7	7	704 (Australia)
8	3.7	3.1	0.9	3.2	3.7	3.8	0.6		96.7	96.7	96.7	96.4	99.2	96.3	96.5	**8**	TR-20 (Japan)
9	1.7	0.3	3.7	0.9	1.7	1.7	3.3	3.3		98.5	98.4	98.1	96.4	98.5	98.4	**9**	BD-3 (Bangladesh)
10	1.4	1.1	3.5	1.3	1.4	1.4	3.1	3.2	1.5		99.0	98.9	96.5	99.1	99.1	**10**	98D02152 (USA)
11	1.4	1.3	3.7	1.4	1.4	1.5	3.2	3.4	1.5	1.0		98.6	96.6	98.7	98.7	**11**	Harbin (China)
12	1.7	1.5	3.8	1.7	1.6	1.7	3.4	3.5	1.9	1.1	1.4		96.3	98.6	99.8	**12**	SMSC-1P60 (Malaysi
13	4.0	3.3	1.0	3.5	4.0	3.8	0.5	0.8	3.7	3.5	3.5	3.8		96.1	96.4	**13**	SMSC-1 (Malaysia)
14	1.7	1.4	3.9	1.7	1.7	1.7	3.6	3.7	1.5	0.9	1.3	1.4	4.0		98.9	**14**	Del Ros (USA)
15	1.5	1.3	3.7	1.4	1.5	1.5	3.3	3.4	1.7	0.9	1.3	0.2	3.7	1.1		**15**	A2 (Japan)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Table. 4. Percent nucleotide similarity and divergence based on the nucleotide sequences of 1766 bp region of different chicken anaemia viruses.

Fig. 14. Alignment of deduced amino acid sequences of VP1 gene region of different chicken anaemia viruses. Aminoacid residues differing from maiority are boxed.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1 MARRARRPRGRFYAFRRGRWHHLKRLRRRYKFRHRRRQRY TR-20 (Japan)
1 MARRARRPRGRFYAFRRGRWHHLKRLRRRYKFRHRRRQRY 98D02152 (USA)
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41 RRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGIIFLTE CAV -E. (India)
41 RRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGIIFLTE CAV -B (India)
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41 RRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGIIFLTE 704 (Australia)
41 RRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGVIFLTE 26P4 (Netherlands)
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41 RRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGVIFLTE Del Ros (USA)
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41 RRRAFRKAFHNPRPGTYSVRLPNPQSTMTIRFQGIIFLTE SMSC-1 (Malaysia)
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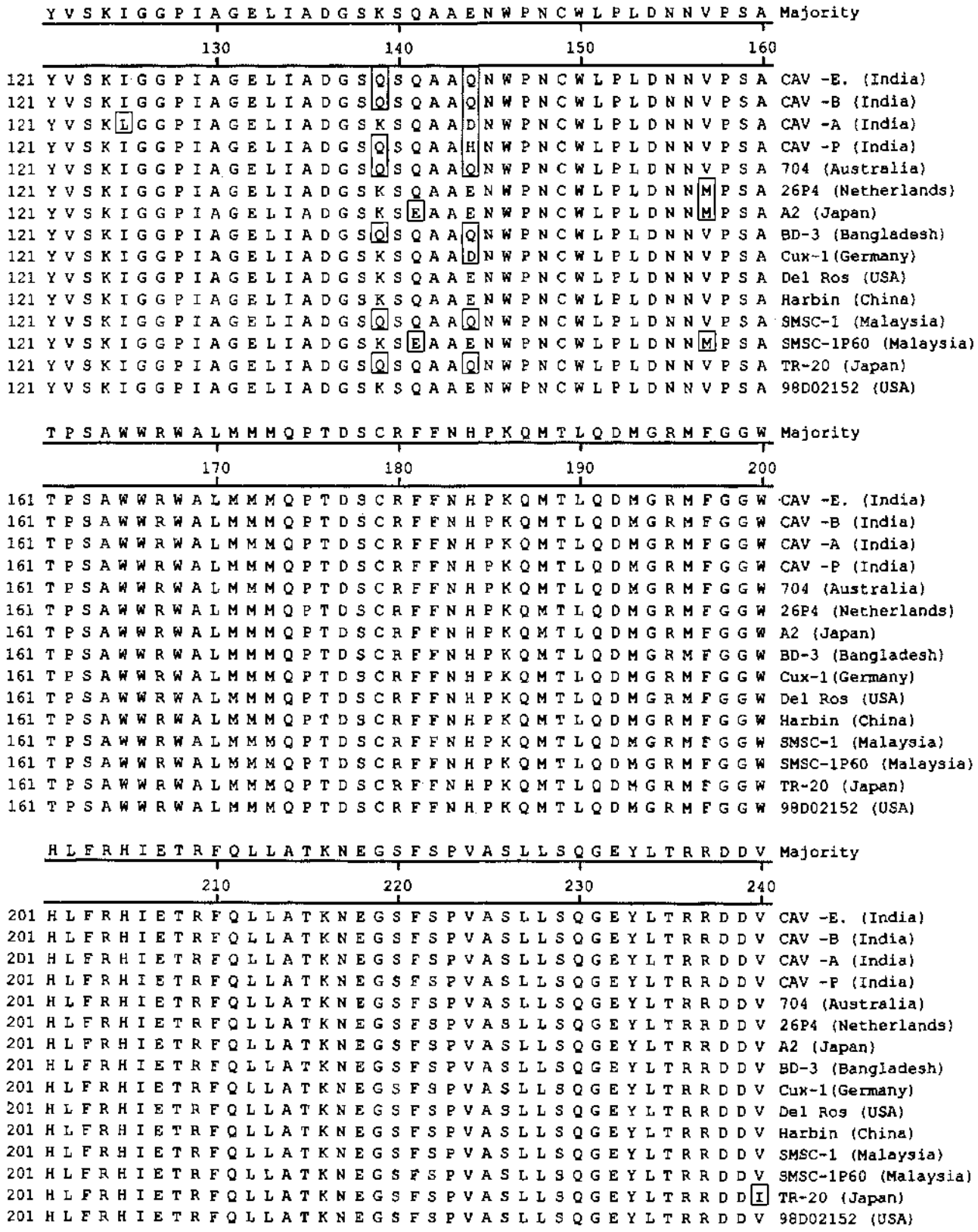
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121 YVSKIGGPIAGELIADGSKSQAAENWPNCWLPLDNNVPSA Del Ros (USA)
121 YVSKIGGPIAGELIADGSKSQAAENWPNCWLPLDNNVPSA Harbin (China)
121 YVSKIGGPIAGELIADGSQSQAAQNWPNCWLPLDNNVPSA SMSC-1 (Malaysia)
121 YVSKIGGPIAGELIADGSKSEAAENWPNCWLPLDNNMPSA SMSC-1P60 (Malaysia)
121 YVSKIGGPIAGELIADGSQSQAAQNWPNCWLPLDNNVPSA TR-20 (Japan)
121 YVSKIGGPIAGELIADGSKSQAAENWPNCWLPLDNNVPSA 98D02152 (USA)
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170
180
190
200
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW CAV -E. (India)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW CAV -B (India)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW CAV -A (India)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW CAV -P (India)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW 704 (Australia)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW 26P4 (Netherlands)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW A2 (Japan)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW BD-3 (Bangladesh)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW Cux-1(Germany)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW Del Ros (USA)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW Harbin (China)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW SMSC-1 (Malaysia)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW SMSC-1P60 (Malaysia)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW TR-20 (Japan)
161 TPSAWWRWALMMMQPTDSCRFFNHPKQMTLQDMGRMFGGW 98D02152 (USA)
HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV Majority
210
220
230
240
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV CAV -E. (India)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV CAV -B (India)
2D1 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV CAV -A (India)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV CAV -P (India)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV 704 (Australia)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV 26P4 (Netherlands)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV A2 (Japan)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV BD-3 (Bangladesh)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV Cux-1(Germany)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV Del Ros (USA)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV Harbin (China)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV SMSC-1 (Malaysia)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDV SMSC-1P60 (Malaysia)
201 HLFRHIETRFQLLATKNEGSFSPVASLLSQGEYLTRRDDI TR-20 (Japan)
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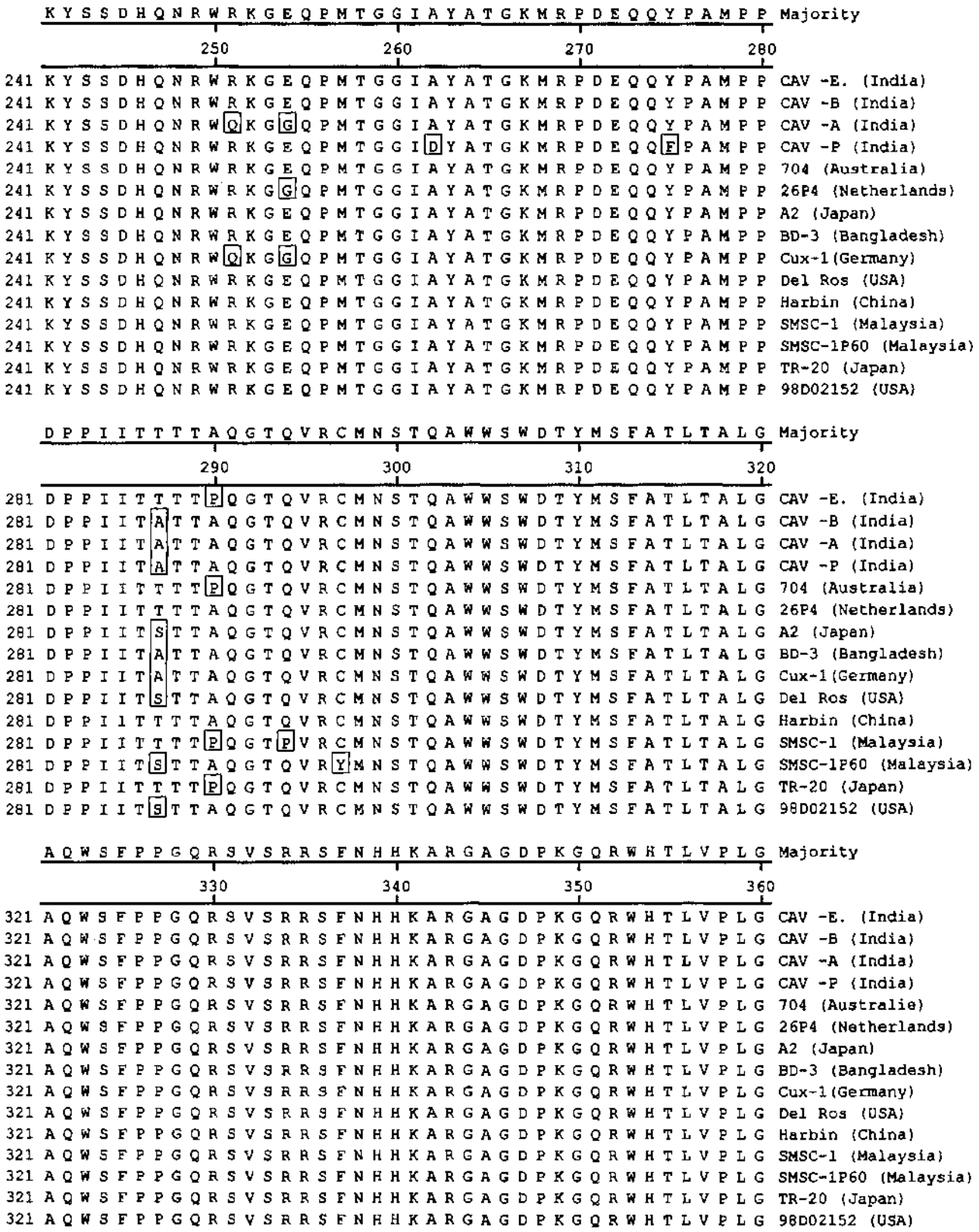
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260
270
280
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241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P CAV -B (India)
241 K Y S S D H Q N R W Q K G G Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P CAV -A (India)
241 K Y S S D H Q N R W R K G E Q P M T G G I D Y A T G K M R P D E Q Q F P A M P P CAV -P (India)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P 704 (Australia)
241 K Y S S D H Q N R W R K G G Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P 26P4 (Netherlands)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P A2 (Japan)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P BD-3 (Bangladesh)
241 K Y S S D H Q N R W Q K G G Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P Cux-1(Germany)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P Del Ros (USA)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P Harbin (China)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P SMSC-1 (Malaysia)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P SMSC-1P60 (Malaysia)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P TR-20 (Japan)
241 K Y S S D H Q N R W R K G E Q P M T G G I A Y A T G K M R P D E Q Q Y P A M P P 98D02152 (USA)
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290
300
310
320
281 D P P I I T T T P Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G CAV -E. (India)
281 D P P I I T A T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G CAV -B (India)
281 D P P I I T A T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G CAV -A (India)
281 D P P I I T A T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G CAV -P (India)
281 D P P I I T T T T P Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G 704 (Australia)
281 D P P I I T T T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G 26P4 (Netherlands)
281 D P P I I T S T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G A2 (Japan)
281 D P P I I T A T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G BD-3 (Bangladesh)
281 D P P I I T A T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G Cux-1(Germany)
281 D P P I I T S T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G Del Ros (USA)
281 D P P I I T T T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G Harbin (China)
281 D P P I I T T T T P Q G T P V R C M N S T Q A W W S W D T Y M S F A T L T A L G SMSC-1 (Malaysia)
281 D P P I I T S T T A Q G T Q V R Y M N S T Q A W W S W D T Y M S F A T L T A L G SMSC-1P60 (Malaysia)
281 D P P I I T T T T P Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G TR-20 (Japan)
281 D P P I I T S T T A Q G T Q V R C M N S T Q A W W S W D T Y M S F A T L T A L G 98D02152 (USA)
A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G Majority
330
340
350
360
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G CAV -E. (India)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G CAV -B (India)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G CAV -A (India)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G CAV -P (India)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G 704 (Australia)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G 26P4 (Netherlands)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G A2 (Japan)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G BD-3 (Bangladesh)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G Cux-1(Germany)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G Del Ros (USA)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G Harbin (China)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G SMSC-1 (Malaysia)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G SMSC-1P60 (Malaysia)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G TR-20 (Japan)
321 A Q W S F P P G Q R S V S R R S F N H H K A R G A G D P K G Q R W H T L V P L G 98D02152 (USA)

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    T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  Majority
                      370                 380                 390                 400
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  CAV -E. (India)
361 T E T I T D S Y M [T] A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  CAV -B (India)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  CAV -A (India)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  CAV -P (India)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  704 (Australia)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  26P4 (Netherlands)
361 T E T I T D S Y M [G] A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  A2 (Japan)
361 T E T I T D S Y M [T] A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  BD-3 (Bangladesh)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  Cux-1(Germany)
361 T E T I T D S Y M [G] A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  Del Ros (USA)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  Harbin (China)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  SMSC-1 (Malaysia)
361 T E T I T D S Y M [G] A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  SMSC-1P60 (Malaysia)
361 T E T I T D S Y M S A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  TR-20 (Japan)
361 T E T I T D S Y M [G] A P A S E L D T N F F T L Y V A Q G T N K S Q Q Y K F G T A  98D02152 (USA)

    T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  Majority
                      410                 420                 430
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  CAV -E. (India)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  CAV -B (India)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  CAV -A (India)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  CAV -P (India)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  704 (Australia)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  26P4 (Netherlands)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  A2 (Japan)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  BD-3 (Bangladesh)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  Cux-1(Germany)
401 T Y A L K E P V M K S D [S] W A V V R V Q S V W Q L G N R Q R P  Del Ros (USA)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  Harbin (China)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  SMSC-1 (Malaysia)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  SMSC-1P60 (Malaysia)
401 T Y A L K E P V M K S D A W A V V R V Q S V W Q L G N R Q R P  TR-20 (Japan)
401 T Y A L K E P V M K S D [S] W A V V R V Q S V W Q L G N R Q R P  98D02152 (USA)
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Decoration 'Decoration #1': Box residues that differ from the Consensus.

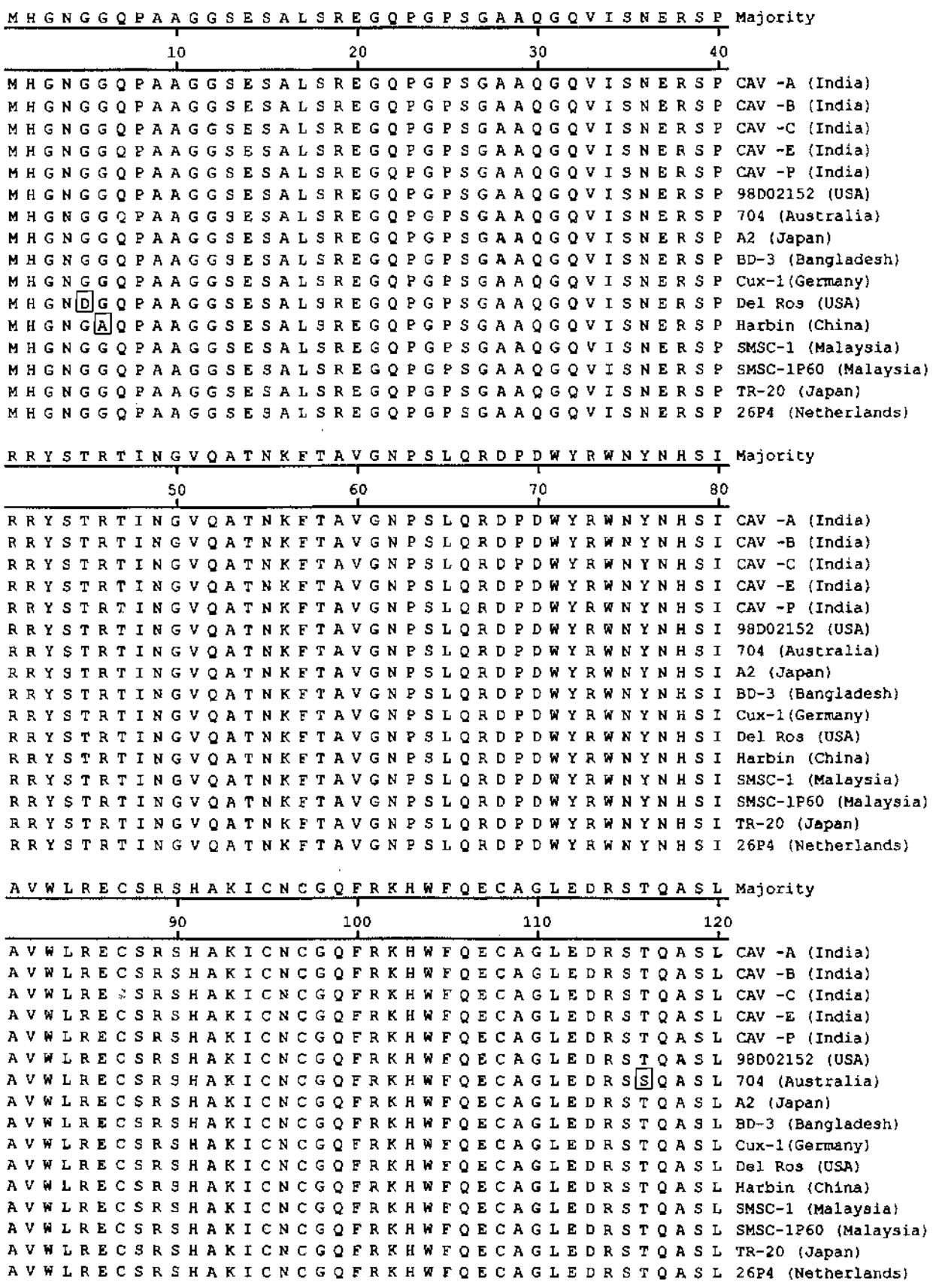
Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		99.3	97.9	98.8	100.0	98.4	97.9	99.3	97.9	98.1	98.8	99.5	97.4	99.8	98.1	**1**	CAV -E. (India)
2	0.7		98.1	99.1	99.3	98.1	98.1	100.0	98.1	98.4	98.6	98.8	97.7	99.1	98.4	**2**	CAV -B (India)
3	2.1	1.9		97.9	97.9	98.8	98.1	98.1	99.5	98.4	98.8	97.4	97.7	97.7	98.4	**3**	CAV -A (India)
4	1.2	0.9	2.1		98.8	97.9	97.7	99.1	97.9	97.9	98.4	98.4	97.2	98.6	97.9	**4**	CAV -P (India)
5	0.0	0.7	2.1	1.2		98.4	97.9	99.3	97.9	98.1	98.8	99.5	97.4	99.8	98.1	**5**	704 (Australia)
6	1.6	1.9	1.2	2.1	1.6		99.1	98.1	98.8	98.8	99.5	97.9	98.6	98.1	98.8	**6**	26P4 (Netherlands)
7	2.1	1.9	1.9	2.4	2.1	0.9		98.1	98.1	99.3	99.1	97.4	99.5	97.7	99.3	**7**	A2 (Japan)
8	0.7	0.0	1.9	0.9	0.7	1.9	1.9		98.1	98.4	98.6	98.8	97.7	99.1	98.4	**8**	BD-3 (Bangladesh)
9	2.1	1.9	0.5	2.1	2.1	1.2	1.9	1.9		98.4	98.8	97.4	97.7	97.7	98.4	**9**	Cux-1(Germany)
10	1.9	1.6	1.6	2.1	1.9	1.2	0.7	1.6	1.6		99.3	97.7	98.8	97.9	100.0	**10**	Del Ros (USA)
11	1.2	1.4	1.2	1.6	1.2	0.5	0.9	1.4	1.2	0.7		98.4	98.6	98.6	99.3	**11**	Harbin (China)
12	0.5	1.2	2.6	1.6	0.5	2.1	2.6	1.2	2.6	2.4	1.6		97.0	99.3	97.7	**12**	SMSC-1 (Malaysia)
13	2.6	2.4	2.4	2.8	2.6	1.4	0.5	2.4	2.4	1.2	1.4	3.1		97.2	98.8	**13**	SMSC-1P60 (Malaysia
14	0.2	0.9	2.4	1.4	0.2	1.9	2.4	0.9	2.4	2.1	1.4	0.7	2.8		97.9	**14**	TR-20 (Japan)
15	1.9	1.6	1.6	2.1	1.9	1.2	0.7	1.6	1.6	0.0	0.7	2.4	1.2	2.1		**15**	98D02152 (USA)
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**		

Table. 5. Percent nucleotide similarity and divergence based on the amino acid sequences of VP1 region of different chicken anaemia viruses.

Fig. 15. Alignment of deduced amino acid sequences of VP2 gene region of different chicken anaemia viruses. Aminoacid residues differing from majority are boxed.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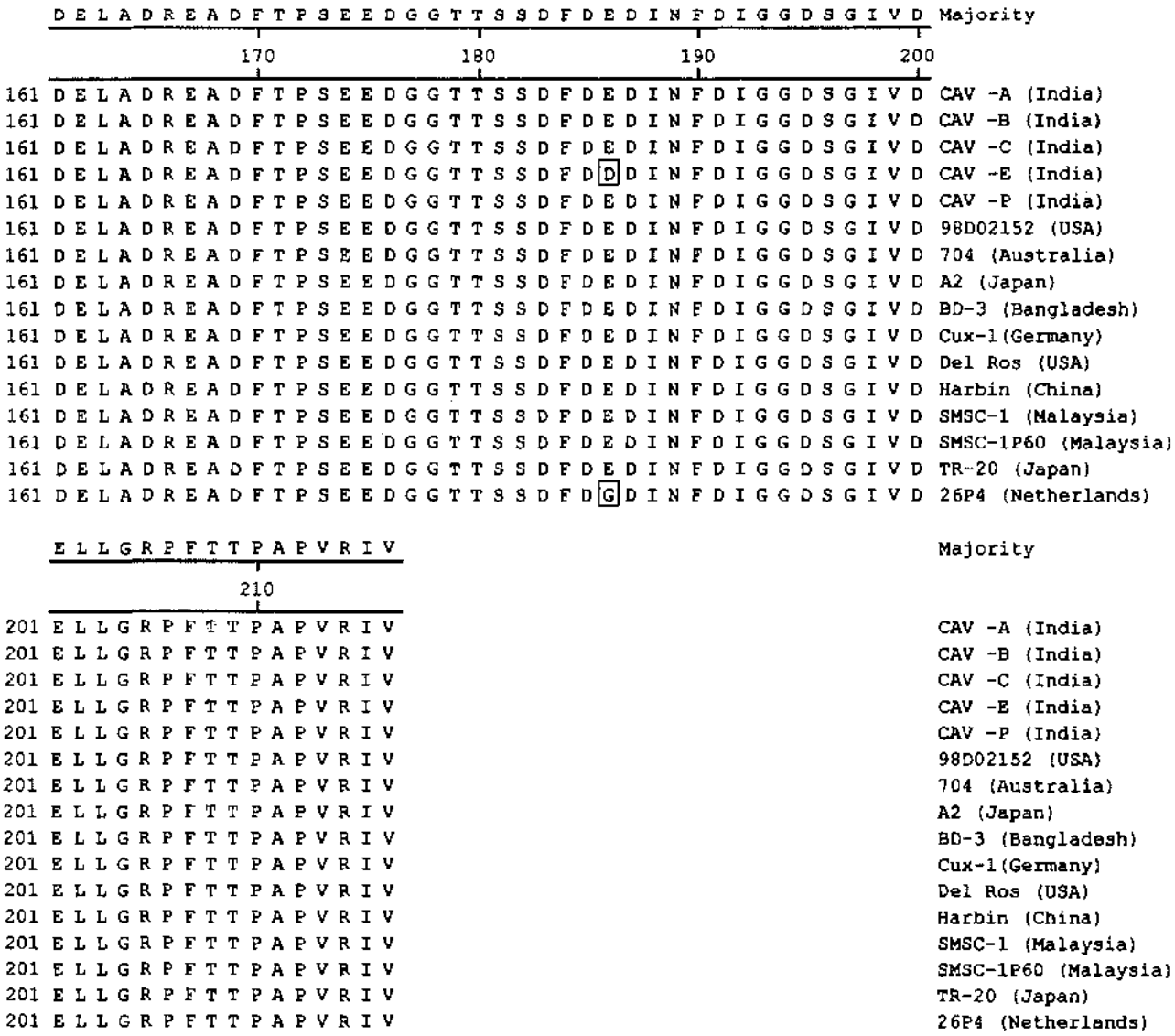
10
20
30
40
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP CAV -A (India)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP CAV -B (India)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP CAV -C (India)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP CAV -E (India)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP CAV -P (India)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP 98D02152 (USA)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP 704 (Australia)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP A2 (Japan)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP BD-3 (Bangladesh)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP Cux-1(Germany)
1 MHGNDGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP Del Ros (USA)
1 MHGNAGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP Harbin (China)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP SMSC-1 (Malaysia)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP SMSC-1P60 (Malaysia)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP TR-20 (Japan)
1 MHGNGGQPAAGGSESALSREGQPGPSGAAQGQVISNERSP 26P4 (Netherlands)
RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI Majority
50
60
70
80
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI CAV -A (India)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI CAV -B (India)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI CAV -C (India)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI CAV -E (India)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI CAV -P (India)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI 98D02152 (USA)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI 704 (Australia)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI A2 (Japan)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI BD-3 (Bangladesh)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI Cux-1(Germany)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI Del Ros (USA)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI Harbin (China)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI SMSC-1 (Malaysia)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI SMSC-1P60 (Malaysia)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI TR-20 (Japan)
41 RRYSTRTINGVQATNKFTAVGNPSLQRDPDWYRWNYNHSI 26P4 (Netherlands)
AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL Majority
90
100
110
120
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL CAV -A (India)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL CAV -B (India)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL CAV -C (India)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL CAV -E (India)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL CAV -P (India)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL 98D02152 (USA)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSSQASL 704 (Australia)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL A2 (Japan)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL BD-3 (Bangladesh)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL Cux-1(Germany)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL Del Ros (USA)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL Harbin (China)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL SMSC-1 (Malaysia)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL SMSC-1P60 (Malaysia)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL TR-20 (Japan)
81 AVWLRECSRSHAKICNCGQFRKHWFQECAGLEDRSTQASL 26P4 (Netherlands)



Decoration 'Decoration #1': Box residues that differ from the Consensus.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1		99.5	99.5	99.1	99.5	99.5	99.1	99.5	99.5	99.1	99.1	99.1	99.5	99.1	99.5	99.1	**1**	CAV -A (India)
2	0.5		100.0	99.5	100.0	100.0	99.5	100.0	100.0	99.5	99.5	99.5	100.0	99.5	100.0	99.5	**2**	CAV -B (India)
3	0.5	0.0		99.5	100.0	100.0	99.5	100.0	100.0	99.5	99.5	99.5	100.0	99.5	100.0	99.5	**3**	CAV -C (India)
4	0.9	0.5	0.5		99.5	99.5	99.1	99.5	99.5	99.1	99.1	99.1	99.5	99.1	99.5	99.5	**4**	CAV -E (India)
5	0.5	0.0	0.0	0.5		100.0	99.5	100.0	100.0	99.5	99.5	99.5	100.0	99.5	100.0	99.5	**5**	CAV -P (India)
6	0.5	0.0	0.0	0.5	0.0		99.5	100.0	100.0	99.5	99.5	99.5	100.0	99.5	100.0	99.5	**6**	98D02152 (USA)
7	0.9	0.5	0.5	0.9	0.5	0.5		99.5	99.5	99.1	99.1	99.1	99.5	99.1	99.5	99.1	**7**	704 (Australia)
8	0.5	0.0	0.0	0.5	0.0	0.0	0.5		100.0	99.5	99.5	99.5	100.0	99.5	100.0	99.5	**8**	A2 (Japan)
9	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0		99.5	99.5	99.5	100.0	99.5	100.0	99.5	**9**	BD-3 (Bangladesh)
10	0.9	0.5	0.5	0.9	0.5	0.5	0.9	0.5	0.5		99.1	99.1	99.5	100.0	99.5	99.1	**10**	Cux-1(Germany)
11	0.9	0.5	0.5	0.9	0.5	0.5	0.9	0.5	0.5	0.9		99.1	99.5	99.1	99.5	99.1	**11**	Del Ros (USA)
12	0.9	0.5	0.5	0.9	0.5	0.5	0.9	0.5	0.5	0.9	0.9		99.5	99.1	99.5	99.1	**12**	Harbin (China)
13	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.5	0.5		99.5	100.0	99.5	**13**	SMSC-1 (Malaysia)
14	0.9	0.5	0.5	0.9	0.5	0.5	0.9	0.5	0.5	0.0	0.9	0.9	0.5		99.5	99.1	**14**	SMSC-1P60 (Malaysi
15	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0	0.5	0.5	0.5	0.0	0.5		99.5	**15**	TR-20 (Japan)
16	0.9	0.5	0.5	0.5	0.5	0.5	0.9	0.5	0.5	0.9	0.9	0.9	0.5	0.9	0.5		**16**	26P4 (Netherlands)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		

Table. 6. Percent nucleotide similarity and divergence based on the amino acid sequences of VP2 region of different chicken anaemia viruses.

ranging from 0 to 0.9% among the Indian and foreign isolates (Table 6). Among the Indian isolates CAV-E showed maximum variation of 0.9%. CAV-B, CAV-C and CAV-P were 100% identical among themselves and also with foreign strains viz., BD-3 (Bangladesh), TR-20 (Japan) and SMSC-P (Malaysia).

2.5 Phylogenetic analysis

Phylogenetic analysis based on the nucleotide sequence of 1766 bp region of Indian CAV isolates along with foreign isolates is given in Fig.16. This study revealed that CAV-A and Cux-1 had similar lineage and very closely related since they formed a cluster whereas CAV-B and CAV-P formed a separate cluster along with the BD-3 (Bangladesh). CAV-E formed a different cluster along with 704 (Australian), TR-20 (Japanese) and SMSC-1 (Malaysian) isolates which had a separate unique lineage. The USA isolates (Del Ross, DS2152), Japanese (A-2) and Malaysian attenuated SMSC-1 P60 strain formed another separate cluster.

Phylogenetic tree based on the 431 amino acids sequence of VP1 (Fig. 17) indicated that CAV-A was closely related to European whereas CAV-B, P and E were closely related to Australian, Malaysian and Bangladesh strains. Phylogenetic analysis based on the amino acid sequences of VP2 region did not reveal any major difference (Fig. 18).

3. PATHOGENICITY STUDIES

Pathogenicity studies in day old SPF chicks indicated that all the five Indian isolates of CAV are highly pathogenic and no significant variation was found among them. The results of the comparative pathogenicity of all the 5 isolates are given in Table 7. All the isolates produced severe anaemia, reduced weight gain, gross and microscopic lesions in the infected chicks. The mean PCV value of CAV infected groups ranged from 12.83±3.32 to 15.33±2.07 as compared to the PCV of control chicks 32.33±3.39 on 15th day post infection. Similarly, the mean body weight of the CAV infected groups was significantly lower (85.0±05.62 to 89.00±4.90) than the control chicks (103.50±5.43) on 15 DPI. Difference among the isolates was found based on the mortality. CAV-E, CAV-P and CAV-A caused death of 2,1 and 1 chicks, respectively. All the Indian isolates invariably produced atrophy of thymus, aplasia of bone marrow, spleen and bursal atrophy, and paleness of liver (Fig. 19). All the isolates also produced severe lymphocyte depletion in the lymphoid organs viz., thymus, bone marrow, spleen and bursa of all the infected chicks (Fig. 20). Few chicks of the infected group that showed lesions in thymus, bone marrow

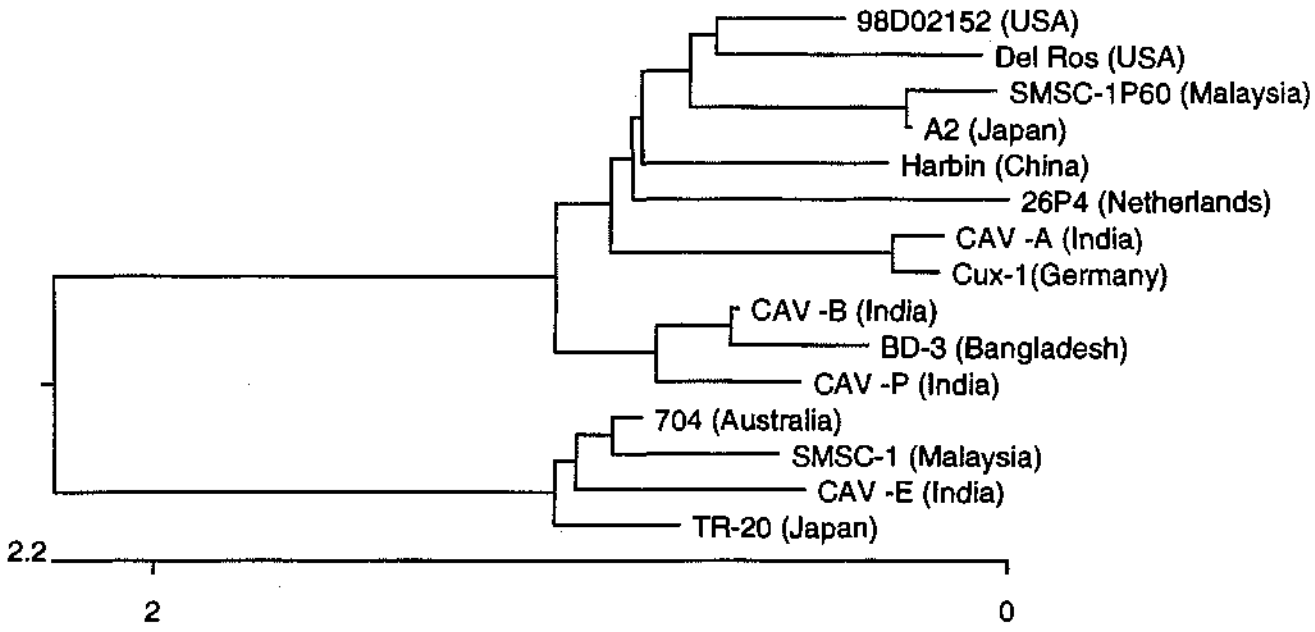


Fig.16. Phylogenetic tree (Cladogram) based on the nucleotide sequences of 1766 bp region of different chicken anaemia viruses. Branch distances corresponded to sequence divergence.

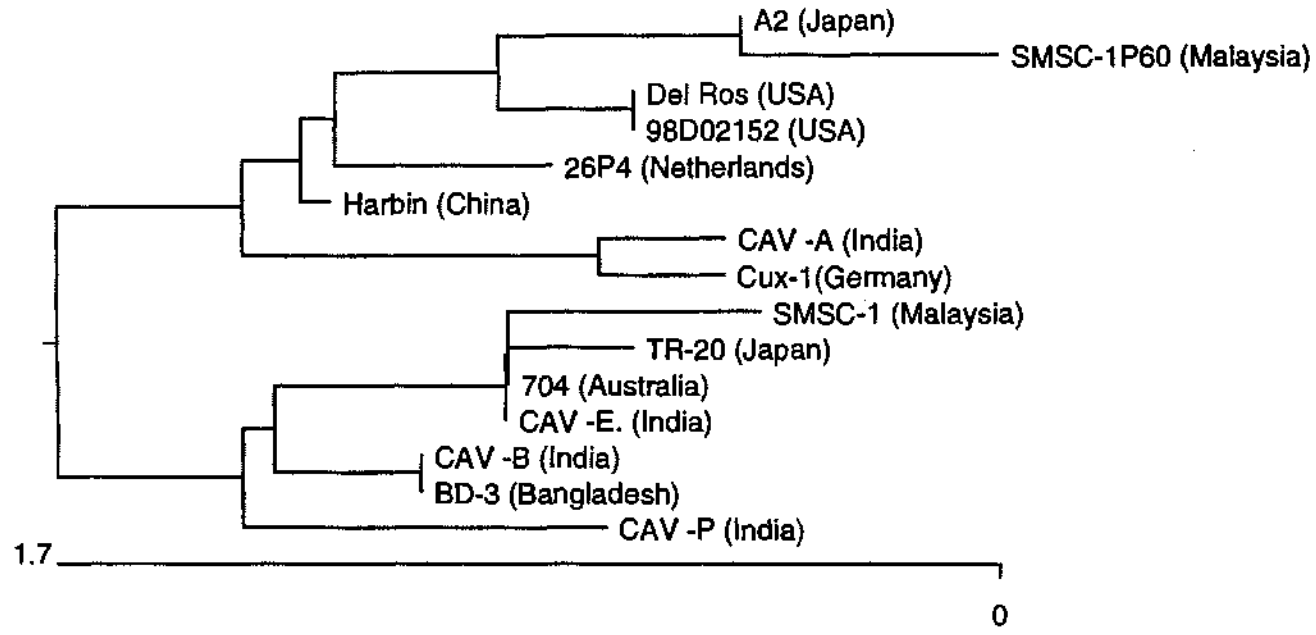


Fig.17. Phylogenetic tree (Cladogram) based on the deduced amino acid sequences of partial VP-1 gene region of different chicken anaemia viruses. Branch distances corresponded to sequence divergence.

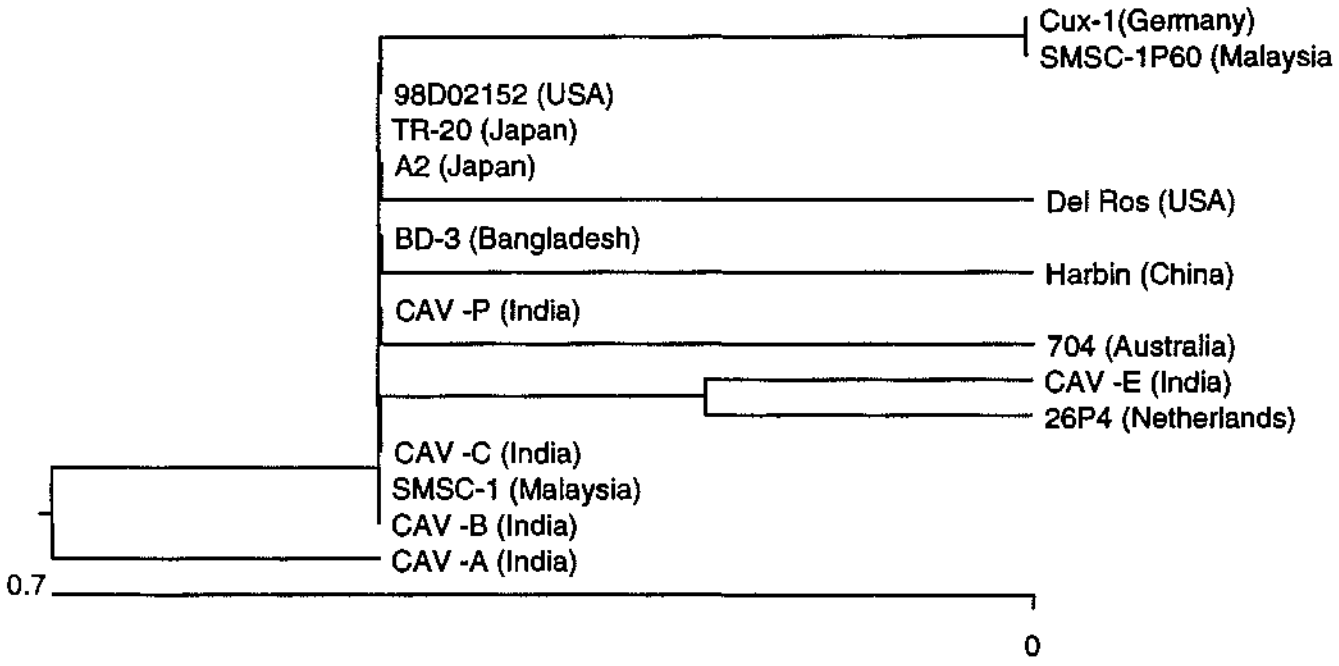


Fig.18. Phylogenetic tree based on the deduced amino acid sequences of complete VP-2 gene region of different chicken anaemia viruses. Branch distances corresponded to sequence divergence.

Table 7 : Clinical signs, mean body weight, mean PCV values, mortality, gross and histopathological lesion of day-old SPF chicks experimentally infected with different CIAV isolates.

Observations	Days post infection	CAV isolates					Control
		CAV-A	CAV-B	CAV-C	CAV-E	CAV-P	
Clinical signs	5	Healthy	Healthy	Healthy	Healthy	Healthy	Healthy
	10	Healthy	Healthy	Healthy	2 chicks- dull depressed	3 chicks were dull	Healthy
	15	2- dull &depressed	Healthy	Healthy	3-dull &depressed	2-depressed	Healthy
PCV (%)	5	28.83±2.32	30.67±2.16	30.67±2.58	31.17±3.06	31.66±2.50	31.00±2.00
(N=6)	10	18.50±1.52^{b}	18.33±2.88^{b}	20.17±2.32^{b}	18.17±3.97^{b}	18.00±3.27^{b}	30.83±2.79^{a}
(Mean±SD)	15	15.33±2.07^{b}	14.33±3.14^{b}	14.67±2.94^{b}	14.83±2.93^{b}	12.83±3.32^{b}	32.33±3.39^{a}
Mean body	5	37.33±11.00^{a}	42.83±±2.13	43.33±2.87	44.33±3.93	42.00±2.28	43.33±2.16^{b}
weight (g)	10	54.50±4.68^{b}	58.00±3.03^{b}	55.50±2.43^{b}	52.16±5.03^{b}	54.33±3.93^{b}	65.50±2.51^{a}
(N=10)	15	85.0±05.62^{b}	88.50±4.46^{b}	88.50±6.47^{b}	89.00±4.90^{b}	88.33±5.57^{b}	103.50±5.43^{a}
(Mean±SD)							
Mortality		1	0	0	2	1	0
Gross lesion							
Thymus		6/10	8/10	5/10	8/10	9/10	0/10
Bone marrow		4/10	5/10	5/10	4/10	7/10	0/10
Liver		9/10	9/10	7/10	8/10	6/10	2/10
Spleen		8/10	6/10	5/10	5/10	7/10	0/10
Bursa		6/10	4/10	3/10	4/10	5/10	0/10
Histopathology							
Thymus		10/10	10/10	10/10	10/10	10/10	0/10
Bone marrow		10/10	10/10	10/10	10/10	10/10	0/10
Liver		8/10	4/10	5/10	4/10	7/10	0/10
Spleen		7/10	8/10	7/10	10/10	10/10	0/10
Bursa		10/10	10/10	10/10	10/10	10/10	0/10

N = Number of chicks

Values with different superscript differs significantly at $P< 0.05$

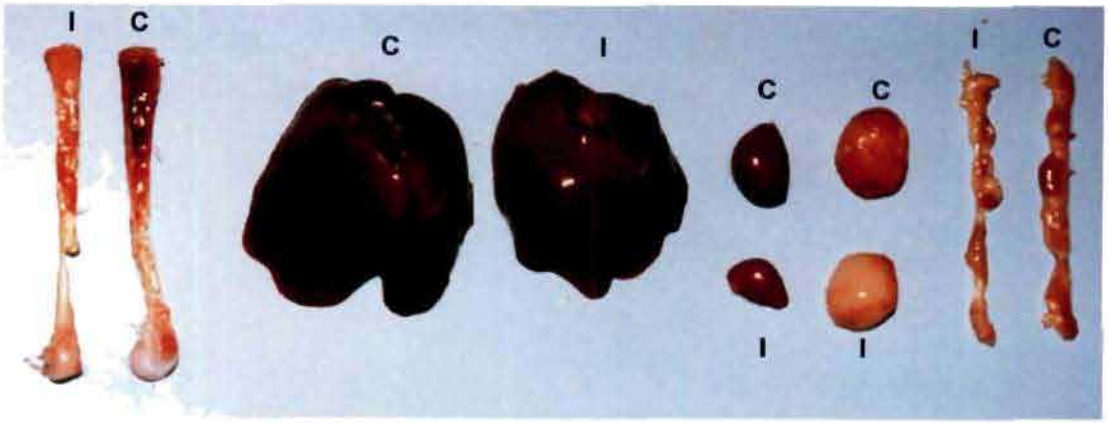
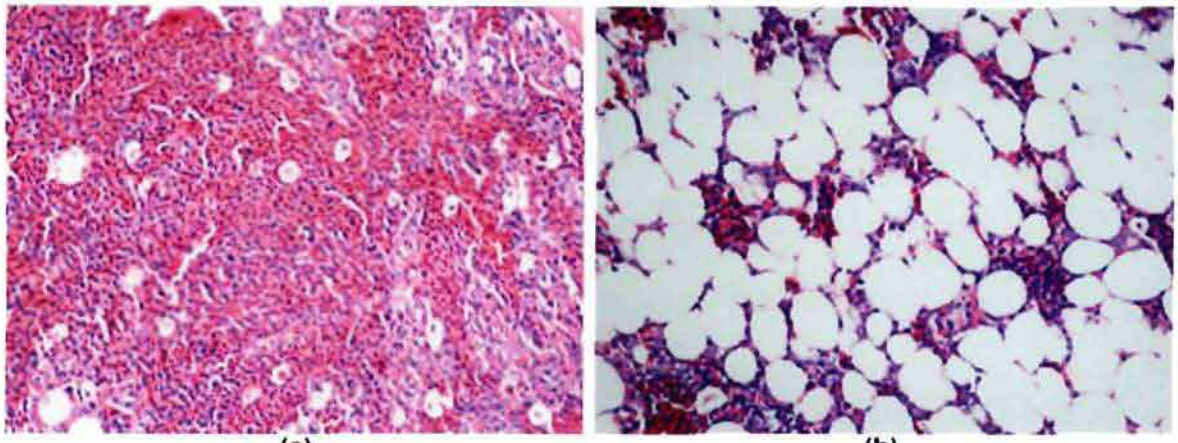
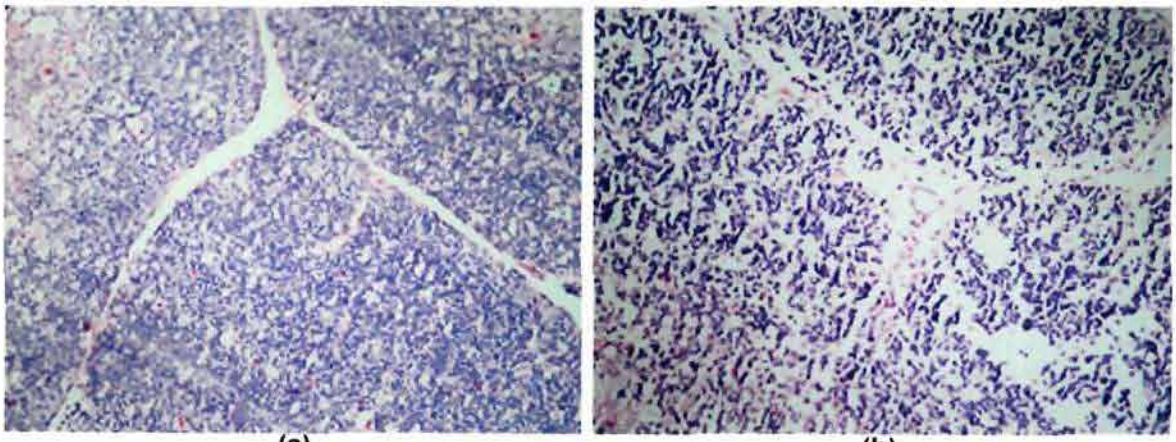


Fig. 19: Gross lesions of different organs of infected chicks along with control (C-Control; I-Infected, organs of infected chicks showing atrophy of thymus, spleen and bursa; palenes of bone marrow and liver).



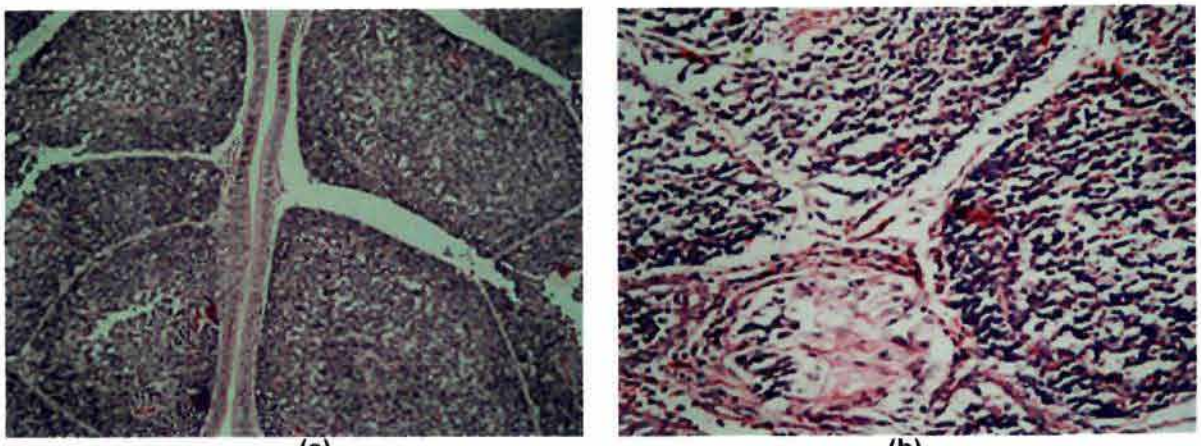
(a) **(b)**

Fig. 20 (i): Infected chicks showing marked aplasia of erythroid and myeloid series of cells with the replacement by lipocytes in bone marrow at 15 DPI (a- control; b-infected). H & E x 400



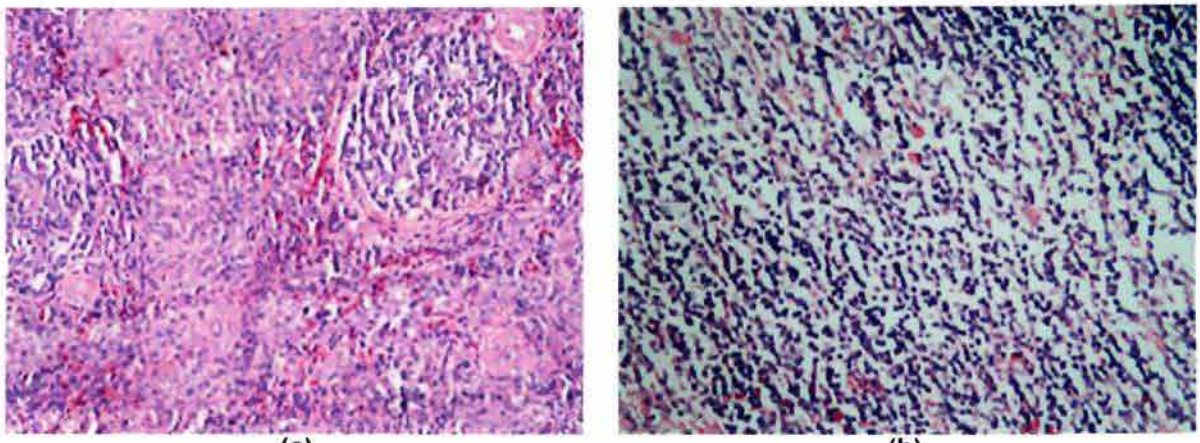
(a) **(b)**

Fig.20 (ii) : Infected chicks showing marked degeneration and depletion of lymphocytes in the cortex and medulla of thymus at 15 DPI (a-control; b-infected). H & E x 400.



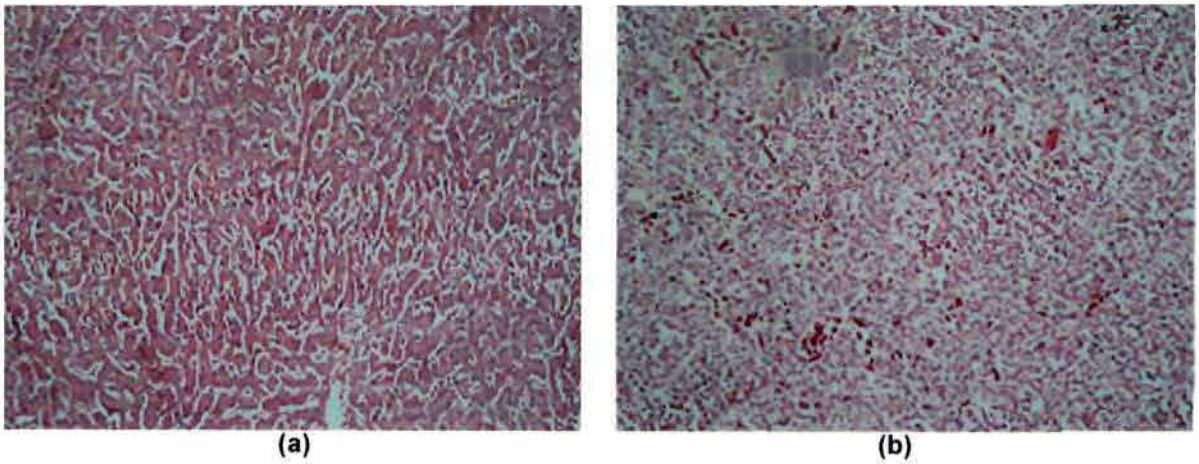
(a) (b)

Fig. 20(iii) : Infected chicks showing degeneration and depletion of lymphocytes both in cortex and medulla of bursa of Fabricius at 15 DPI (a-control; b-infected). H & E x 400.



(a) (b)

Fig.20 (iv) : Infected chicks showing degeneration and necrosis of lymphocytes in spleen at 15 DPI (a-control; b-infected). H & E x 400.



(a) (b)

Fig.20 (v) : Infected chicks showing areas of severely degenerated hepatocytes in liver at 15 DPI (a-control; b-infected). H & E x 400.

and bursa did not show any lesion in the spleen and liver. In CAV-C infected group, chicks were healthy throughout the observation period of 15 days and gross lesions were also observed only lesser number of chicks (data given in Table) than other isolates, however, microscopic changes were observed in lymphoid organs of all the chicks. No significant difference among the isolates of CAV was found with respect to microscopic changes in the lymphoid organs, which indicated their immunosuppressive nature.

4. MOLECULAR CHARACTERIZATION OF CAV-VP1 AND VP2 PROTEINS

4.1 Cloning of CAV-VP1 and VP2 gene in pVAX vector

PCR amplified VP1 and VP2 genes of CAV were first cloned into TOPO-T vector. The presence of VP1 and VP2 genes in TOPO-T vector was confirmed by PCR screening using the forward and reverse primer of the insert (Fig. 21 and 22). Ten colonies of VP1 and 10 colonies of VP2 were picked up and screened by PCR. All the colonies of VP1 and VP2 were found positive for the presence of insert. The insert from the TOPO-T vector was released by *Eco* RI digestion. The released insert was cloned into pVAX vector. The presence of VP1 and VP2 insert in pVAX, in right orientation was confirmed by PCR using the forward T7 promoter primer of the vector and reverse primer of the insert (Fig. 23 and 24). Of the 20 colonies screened, three were found positive for VP1 and two were found positive for the VP2. The PCR positive clones of VP1 and VP2 were further confirmed by restriction endonuclease analysis (Fig. 25). RE analysis of pVAX vector using *Xba* I enzyme yielded a product of ~400 bp as expected for the right orientation. Similarly, RE analysis of pVAX-VP2 using *Pst* I yielded ~300 bp product as expected. Sequencing of insert using the T-7 primer confirmed the presence of gene start in right orientation downstream to the CMV promoter.

4.2 *In vitro* expression of recombinant pVAX containing CAV-VP1 and VP2 protein

The recombinant pVAX plasmid containing VP1 or VP2 was transfected in the eukaryotic cells (Hep-2/CEF) to study the *in vitro* expression. The expression of VP1 and VP2 was assayed by indirect immunofluorescent technique. VP1 expression in Hep-2 cells showed specific perinuclear and intra-nuclear immunofluorescence at 48 hrs post transfection (Fig. 26). Intra-cytoplasmic expression of VP2 in CEF cells at 48 hrs post transfection was observed (Fig. 27). No immunofluorescence could be seen in mock-transfected control cells.

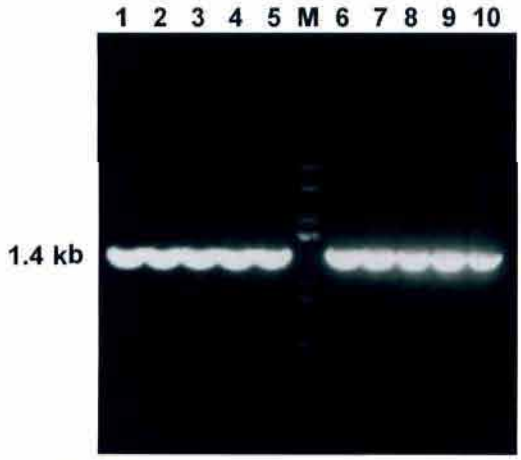


Fig. 21: PCR screening of the bacterial colonies having TOPO-T vector for the presence of CAV-VP1 insert
M: 1 kb DNA ladder (Invitrogen)

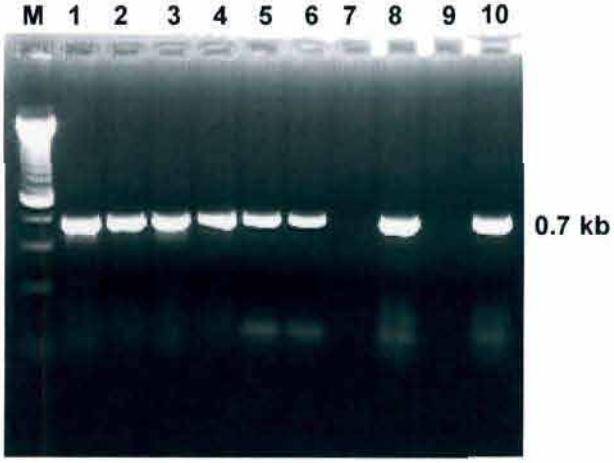


Fig. 22: PCR screening of the bacterial colonies having TOPO-T vector for the presence of CAV-VP2 insert
M: 250 bp DNA ladder (Invitrogen)

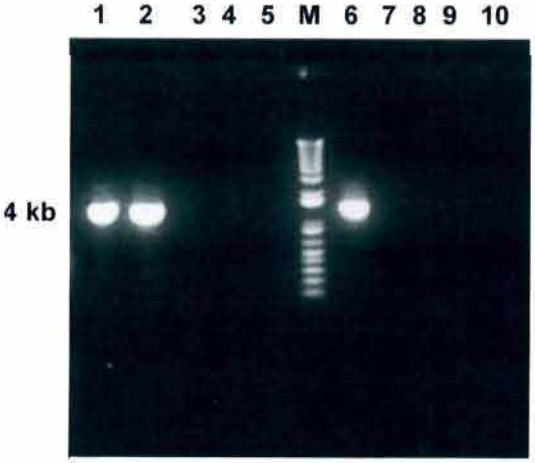


Fig. 23: PCR screening of the bacterial colonies having pVAX vector for the presence of CAV-VP1 insert
M: 1 kb DNA ladder (Invitrogen)

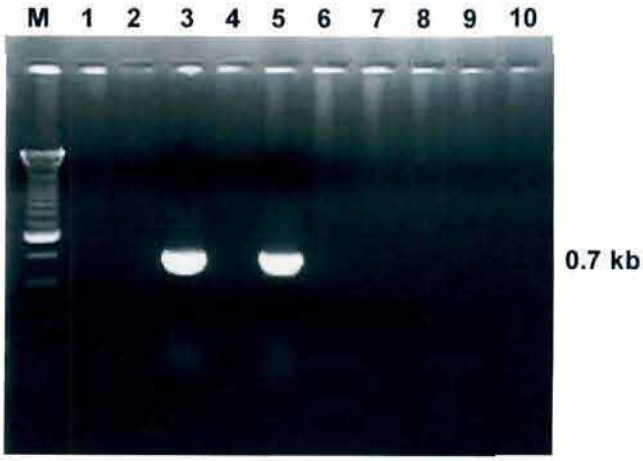
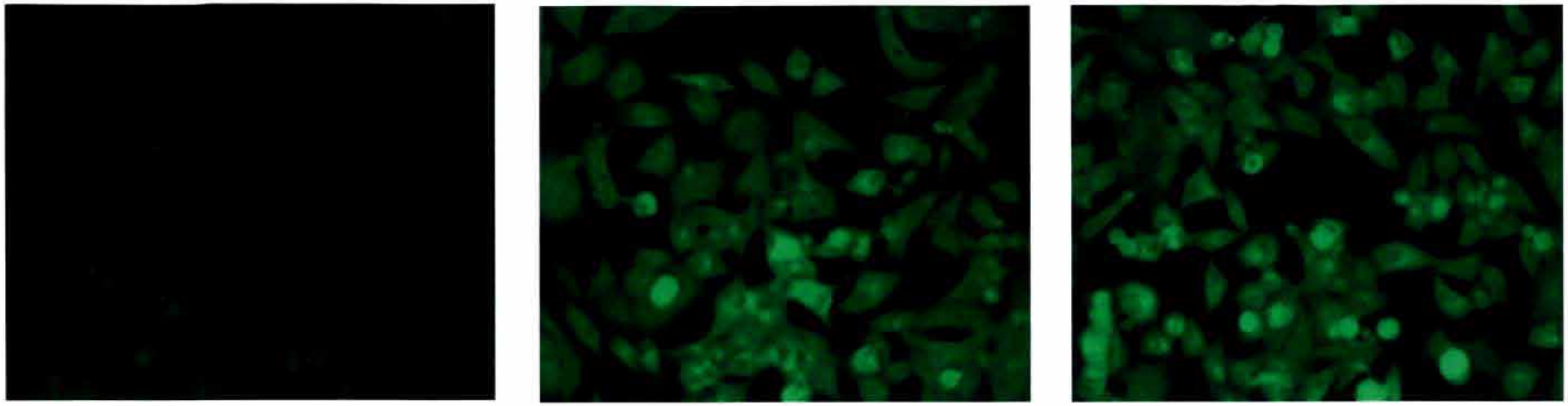


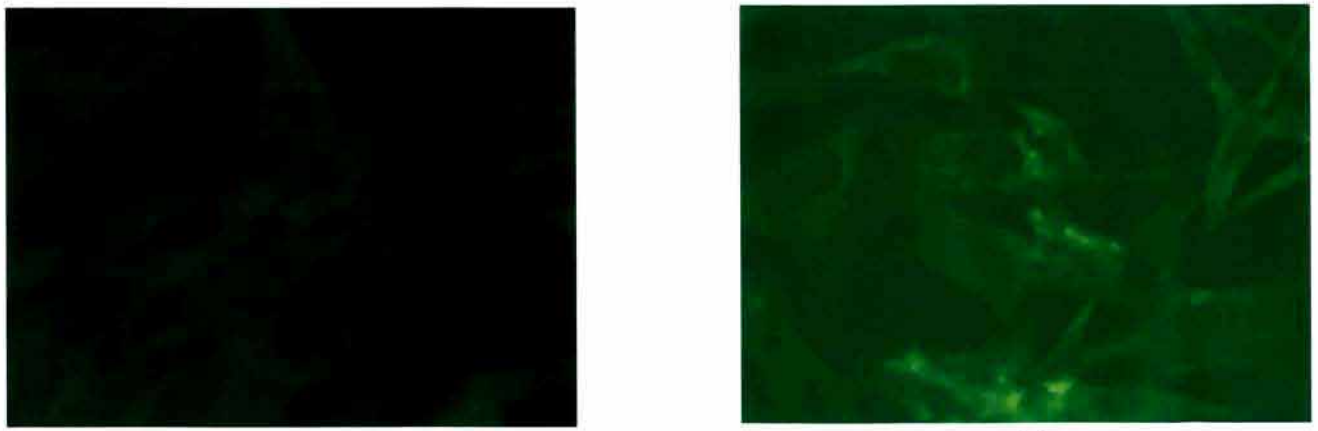
Fig. 24: PCR screening of the bacterial colonies having pVAX vector for the presence of CAV-VP2 insert
M: 250 bp DNA ladder (Invitrogen)

Fig. 25: RE analysis of pVAX-CAV-VP1 using *Xba* I and pVAX-CAV-VP2 using *Pst* I for the confirmation of the right orientation
M: 1 kb DNA ladder



(a) (b) (c)

Fig. 26: Expression of CAV-VP1 in Hep-2 cells showing specific intranuclear and perinuclear immunofluorescence at 48 hr post transfection with pVAX-CAV-VP1. (a) Control; (b) and (c) VP1 transfected. IIFT x 200.



(a) (b)

Fig. 27: Expression of CAV-VP2 in CEF cells showing specific intracytoplasmic immunofluorescence at 48 hr post transfection with pVAX-CAV-VP2. (a) Control; (b) VP2 transfected. IIFT x 400

4.3 Immunization study using pVAX-CAV-VP1 and pVAX-CAV-VP2 in adult birds

The immunogenic and protective effect of CAV-VP1 and VP2 was studied in 18 weeks old adult birds using the recombinant pVAX-CAV-VP1 and pVAX-CAV-VP2 plasmids. The cell mediated and humoral immune response was studied at weekly interval by LTT and ELISA, respectively.

4.3.1 Assessment of cell mediated immune response in immunized adult birds

The cell mediated immune (CMI) response following immunization with CAV-VP1 and VP2 of the different groups (gp 1- control, gp 2- single dose immunization, gp 3- two doses of immunization) of layer birds was assayed by measuring stimulation indices (SI) of peripheral blood mononuclear cells employing LTT using MTT dye at different intervals viz., 7, 14, 21 and 28 days post immunization (Table. 8). The result indicated that CMI response of groups 2 and 3 differed significantly from the control (group 1) on 7 and 14 days post immunization (data given in Table 8). On 21 DPI, group 3 showed significantly higher SI value (1.579±0.096) than the group 1 (1.118±0.091) and 2 (1.311±0.056). In group-2, the highest SI value was found on 14 DPI (1.424±0.056) and thereafter it started declining and was 1.311±0.056 on 21 DPI and 1.191±0.074 on 28 DPI (Fig. 28). In contrast to this, group 3 showed a progressive increase in SI value on 21 DPI (1.579±0.096) and 28 DPI (1.600±0.062).

4.3.2 Assessment of humoral immune response in immunized adult birds

The humoral immune response against CAV was assayed by ELISA at weekly intervals following immunization. The antibody titres of different groups assayed by ELISA are given in Table 9. The antibody titres of all the three groups significantly differed at all the post immunization intervals. The antibody titre in group 2 showed gradual increase and highest titre was detected at 21 DPI (4033.38±76.97) and showed declining trend on 28 DPI (3811.63±52.04) whereas group 3 also showed a gradual increase and maximum titre was detected on 28 DPI (6538.50±86.64) (Fig. 29).

4.3.3 Protection study in young chicks of the immunized dams

The young chicks hatched out from the immunized dams were challenged on 15th day of age and all observations recorded after the challenge virus inoculation are presented in Table 10. Mean PCV value of group 3 on 5 and 10 days post challenge was significantly high (29.00±2.45 and 30.20±2.05) as compared to the group 1 (19.60±2.70 and 14.40±2.41) and 2 (22.60±3.59 and 17.80±1.92). The mean body weight of group 3

Table 8. SI values of LTT assay to assess the CMI response in immunized adult birds

Groups	Days Post Infection (DPI)				
	0	7	14	21	28
I (Control)	1.100±0.061	1.120±0.070[a]	1.123±0.072[a]	1.118±0.091[a]	1.120±0.053[a]
II (Single dose)	1.137±0.111	1.356±0.066[b]	1.424±0.056[b]	1.311±0.056[b]	1.191±0.074[a]
III (Booster dose)	1.104±0.089	1.137±0.086[b]	1.353±0.107[b]	1.579±0.096[c]	1.600±0.062[b]

Values with different superscript differs significantly (row-wise) at $P < 0.05$.

Table 9. Antibody titre values assayed by ELISA to assess the humoral immune response in immunized adult birds

Groups	Days Post Infection				
	0	7	14	21	28
I Control	300.50±59.93	336.38±38.54[a]	373.50±64.15[a]	214.38±48.16[a]	268.75±34.16[a]
II Single	271.25±57.34	1631.25±89.50[b]	3343.25±83.63[b]	4033.38±76.97[b]	3811.63±52.04[b]
III Double	274.00±68.40	1925.63±92.87[c]	3050.88±65.97[c]	5675.00±64.32[c]	6538.50±86.64[c]

Values with different superscript differs significantly (row-wise) at $P < 0.05$.

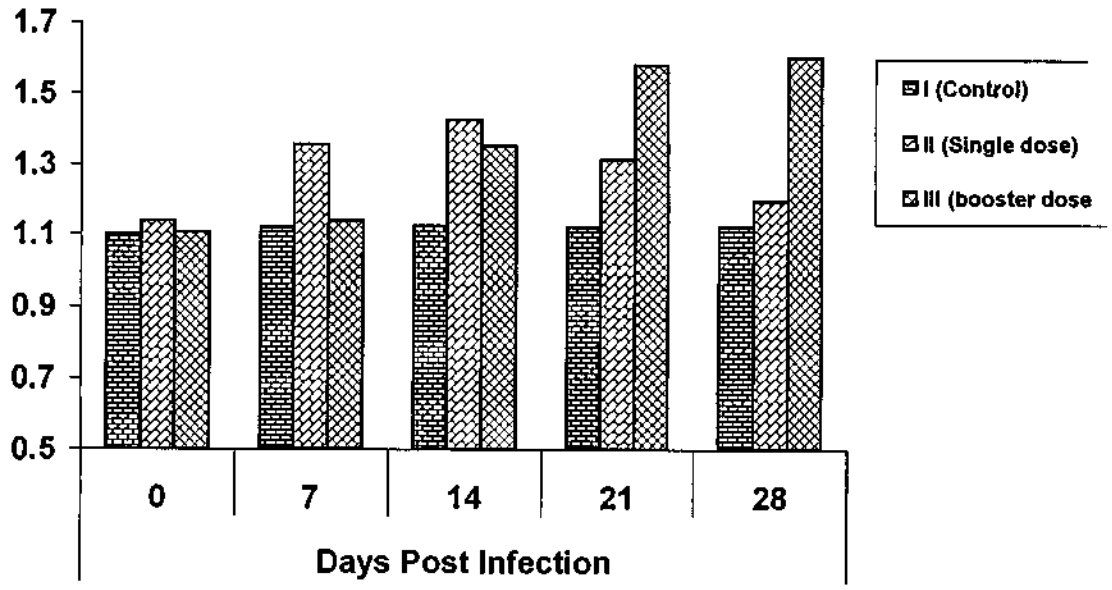


Fig. 28: SI values of LTT assay to assess the CMI response in immunized adult birds

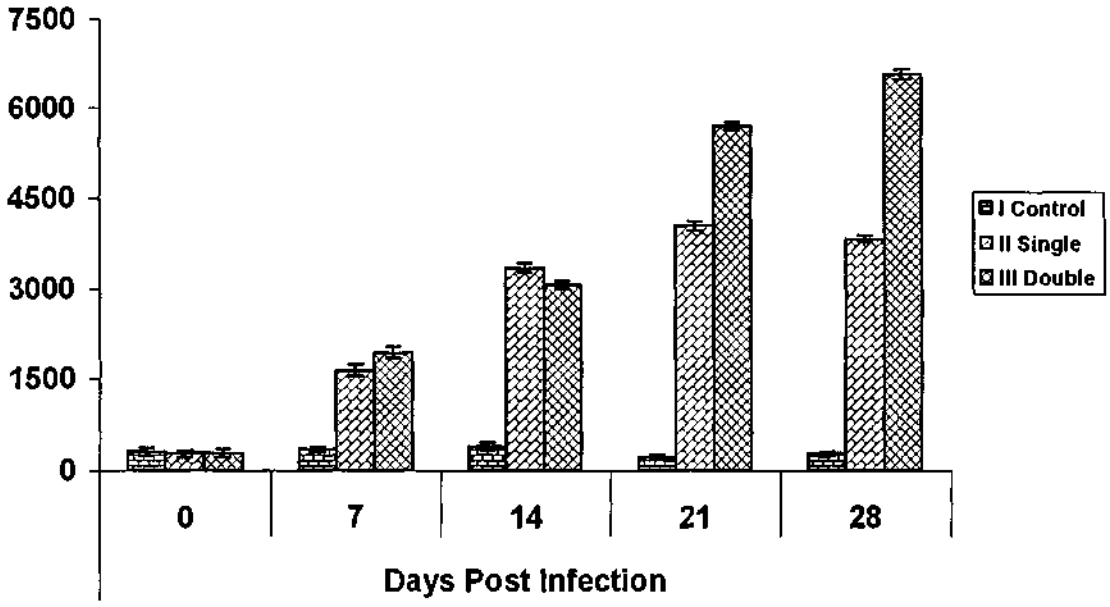


Fig. 29: Antibody titre values assayed by ELISA to assess the humoral immune response in immunized adult birds

Table 10. Clinical signs, mean body weight, mean PCV values, mortality, gross and histopathological lesion of the day old chicks obtained from the immunized dams and challenged with CAV at 15 days age

Observations	Group I (control)	Group I (single)	Group III (2 doses)
Clinical signs			
5DPI	Healthy	Healthy	Healthy
10DPI	2 chicks- slightly dull & depressed.	2 chicks –dull and depressed.	Healthy
15DPI	All the chicks were dull, depressed.	3 chicks dull & depressed	Healthy
PCV (%)(N=6) (Mean±SD)			
5 DPI	30.40±1.82	30.20±2.86	28.40±3.85
10 DPI	19.60±2.70[a]	22.60±3.59[b]	29.00±2.45[b]
15 DPI	14.40±2.41[a]	17.80±1.92[b]	30.20±2.05[c]
Mean body weight (g)(N=10) (Mean±SD)			
5 DPI	42.83±3.21	40.42±3.18	42.83±2.13
10 DPI	52.50±2.43[a]	54.87±2.56[a]	61.33±2.14[b]
15 DPI	85.36±6.62[a]	87.60±3.23[a]	101.65±6.48[b]
Mortality	2	2	0
Gross lesion			
Thymus atrophy	6/8	5/8	2/10
Bone marrow paleness	4/8	4/8	1/10
Liver paleness	7/8	5/8	3/10
Spleen atrophy	7/8	7/8	2/10
Bursal atrophy	4/8	5/8	0/10
Muscular hemorrhage	0/8	1/8	0/10
Histopathology			
Thymus	8/8	8/8	2/10
Bone marrow	8/8	8/8	2/10
Liver	4/8	3/8	0/10
Spleen	8/8	6/8	0/10
Bursa	8/8	8/8	2/10

Values with different superscript differs significantly (column-wise) at $P< 0.05$.

(61.33±2.14 and 101.65±6.48) was also significantly higher from group 1 (52.50±2.43 and 85.36±6.62) and group 2 (54.87±2.56 and 87.60±3.23) on 10 and 15 DPI. All the ten chicks of group 1 and 2 showed lymphocyte depletion in thymus, bone marrow and bursa where as in group 3, only 2 chicks out of 10 showed depletion. The results revealed that the dams immunized with 2 doses i.e. on day 1 and day 14 with recombinant plasmid conferred complete protection from the clinical disease and partial protection from CAV induced immunosuppression to their offspring.

4.4 *In ovo* immunization

In ovo immunization was carried out in 18-day-old SPF embryos, which were divided into 2 groups (gp1- unimmunized; gp 2- immunized). The immunized embryos were hatched out and monitored for humoral immune response at weekly interval by ELISA and the results are presented in Table 11. The chicks were challenged on 15th day of age and the observations recorded are presented in Table 12.

On 10 and 15 DPI, the group 2 showed mean PCV of 30.60±3.36 and 26.80±3.42, respectively, whereas the group 1 showed low PCV of 23.00±3.39 and 15.60±2.88, respectively. The mean body weight on 10 and 15 DPI was 166.60±6.84 and 182.60±8.04 in group 1 and 157.00±4.90 and 173.80±8.76 in group 2, respectively. In Group 2, only three chicks showed thymus atrophy and liver paleness and histopathological examination revealed depletion of lymphocytes in thymus, bone marrow, bursa and spleen of 3 out of 15 chicks (Table 12). The antibody titre of *in ovo* immunized chicks (group 2) was significantly high at 21 and 28 days of age (1516.80±167.31 and 3081.00±122.78) than the unimmunized (group 1) chicks (3202.10±111.74 and 4482.80±124.30), respectively.

5. ANTI-NEOPLASTIC EFFECT OF CAV-VP3 PROTEIN

5.1. Cloning of the CAV-VP3 in pVAX vector

The PCR amplified CAV-VP3 gene was cloned into TOPO-T vector initially and the obtained clones were screened by PCR for the presence of insert. All the 10 clones screened by PCR were found positive (Fig. 30). The insert released from the TOPO-T vector by *Eco* RI digestion was cloned into *Eco* RI site of pVAX vector. Of the 10 bacterial colonies assumed having the recombinant pVAX vector as screened by PCR using the T-7 promoter primer and insert reverse primer to confirm the right orientation of the gene, only one clone was found positive (Fig. 31). Sequencing of the PCR positive clone using

Table 11. Antibody titre value assayed by ELISA to assess the humoral immune response of *in ovo* immunized young chicks.

Groups	Antibody titre value calculated by ELISA			
	7 DPI	14 DPI	21DPI	28DPI
I (Control)	126.80±23.96	138.70±33.68	1516.80±167.31[a]	3202.10±111.74[a]
II (Vaccinated)	144.80±32.54	142.90±28.71	3081.00±122.78[b]	4482.80±124.30[b]

Values with different superscript differs significantly (row-wise) at P< 0.05.

Table 12. Clinical signs, mean body weight, mean PCV values, mortality, gross and histopathological lesion of *in ovo* immunized chicks challenged at day 15.

Observations	Group I	Group II
Clinical signs		
5 DPI	Healthy	Healthy
10 DPI	1 Healthy	Healthy
15 DPI	2 chicks- dull & depressed	Healthy
PCV (%)(N=5) (Mean±SD)		
5 DPI	30.40±1.81	30.80±3.56
10 DPI	23.00±3.39[a]	30.60±3.36[b]
15 DPI	15.60±2.88[a]	26.80±3.42[b]
Mean body weight (g)(N=10) (Mean±SD)		
5 DPI	124.80±9.37	121.60±6.73
10 DPI	157.00±4.90[a]	166.60±6.84[b]
15 DPI	173.80±8.76[a]	182.60±8.04[b]
Gross lesion		
Thymus atrophy	9/15	3/15
Bone marrow paleness	7/15	0/15
Liver paleness	10/15	3/15
Spleen atrophy	11/15	0/15
Bursal atrophy	6/15	0/15
Muscular hemorrhage	0/15	0/15
Histopathology		
Thymus	13/15	3/15
Bone marrow	10/15	3/15
Liver	10/15	1/15
Spleen	11/15	2/15
Bursa	9/15	3/15

Values with different superscript differs significantly (column-wise) at $P< 0.05$.

the T-7 promoter primer further confirmed the right orientation of the insert and presence of gene start downstream to the CMV promoter.

5.2 *In vitro* expression of recombinant pVAX-CAV-VP3

The expression of CAV-VP3 protein (apoptin) by the recombinant plasmid pVAX-CAV-VP3 was carried out in Hep-2 cells. The pVAX-CAV-VP3 transfected Hep-2 cells showed clear intranuclear immunofluorescence, which confirmed the intranuclear localization of apoptin in transformed cells (Fig. 32) Further, it showed that expression of the apoptin lead to induction of apoptosis, which was also indicated by cytomorphological picture of the cells expressing the VP3 protein. The cells expressing apoptin showed characteristic changes of apoptosis viz. nuclear condensation, fragmentation of chromatin and formation of apoptotic bodies.

5.3 *In vitro* anti-neoplastic effect

In vitro anti-neoplastic effect was studied by analyzing the cytomorphological changes using acridine orange - ethidium bromide staining and fluorescent antibody staining of CEF cells transformed with RSV, which were later transfected with pVAX-CAV-VP3. Fluorescent antibody staining using the CAV-specific polyclonal antisera in VP3 transfected RSV transformed CEF cells showed expression of apoptin inside the nuclei of the transformed cells and cytomorphological changes of apoptosis (Fig. 33). Acridine orange-ethidium bromide staining revealed clear apoptotic changes of nuclear condensation, fragmentation of nuclei and formation of small rounded apoptotic bodies (Fig. 34). The study revealed clear understanding that the CAV-VP3 was expressed in the RSV transformed cells to a high level and induced cell death probably via apoptosis as confirmed by double dye staining and IIFT. No similar changes were observed in control CEF cells transformed with RSV.

5.4. *In vivo* anti-neoplastic effect

The anti-neoplastic effect of the recombinant pVAX-CAV-VP3 plasmid was studied in tumors induced with Rous sarcoma virus in SPF chicks. The study was carried out in 4 groups as mentioned in the materials and methods. Group 2, in which the tumor was induced on day 1 and recombinant plasmid was injected intra-tumoraly indicated tumor suppression due to VP3 induced apoptosis. Grossly, the site of injection of pVAX-CAV-VP3 in the tumor showed contraction, necrosis and formation of scar tissue in the chicks of group 2 (Fig. 35). No such gross and microscopic apoptotic changes were seen in tumors of group 1, 3 and 4.

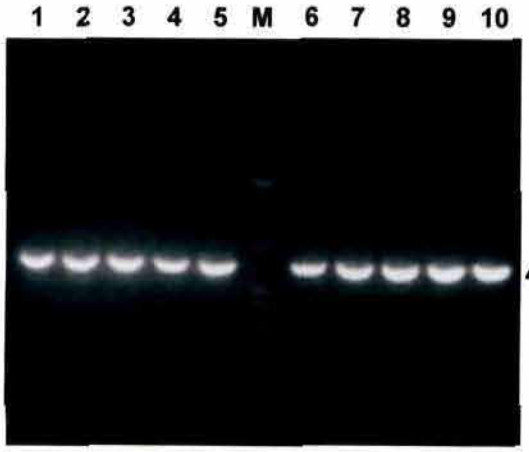


Fig. 30: PCR screening of the bacterial colonies having TOPO-T vector for the presence of CAV-VP3 insert

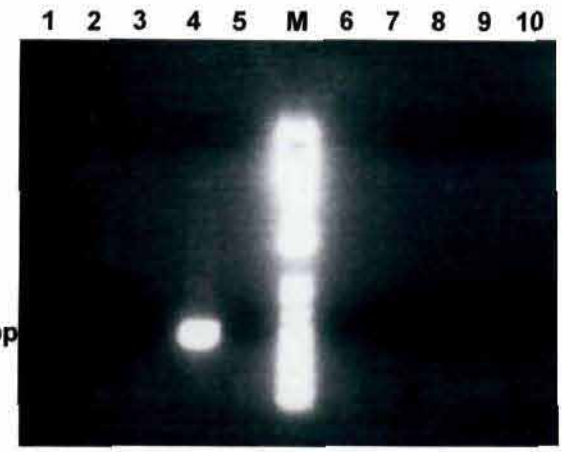
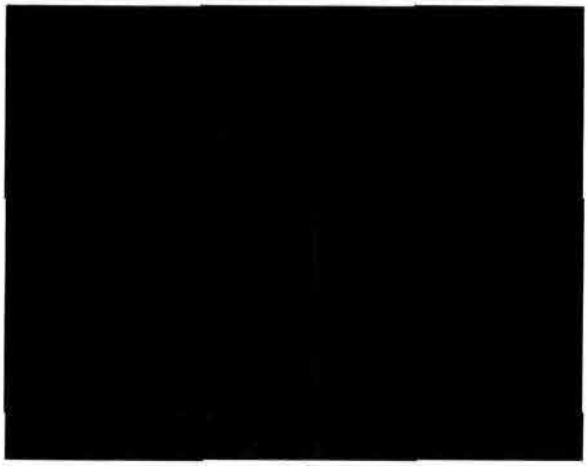
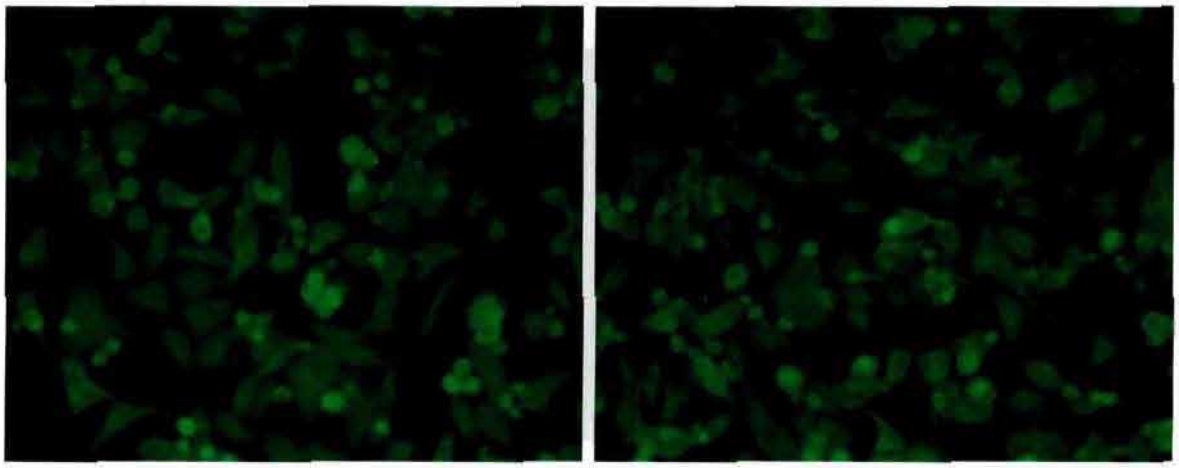


Fig. 31: PCR screening of the bacterial colonies having pVAX vector for the presence of CAV-VP3 insert



(a)



(b) (c)

Fig. 32: Expression of CAV-VP3 in Hep-2 cells showing specific intranuclear immunofluorescence, condensation of nucleus and formation of apoptotic bodies assayed by IIFT at 48 hr post transfection with pVAX-CAV-VP3. (a) Control; (b) and (c) VP3 transfected. IIFT x 200.

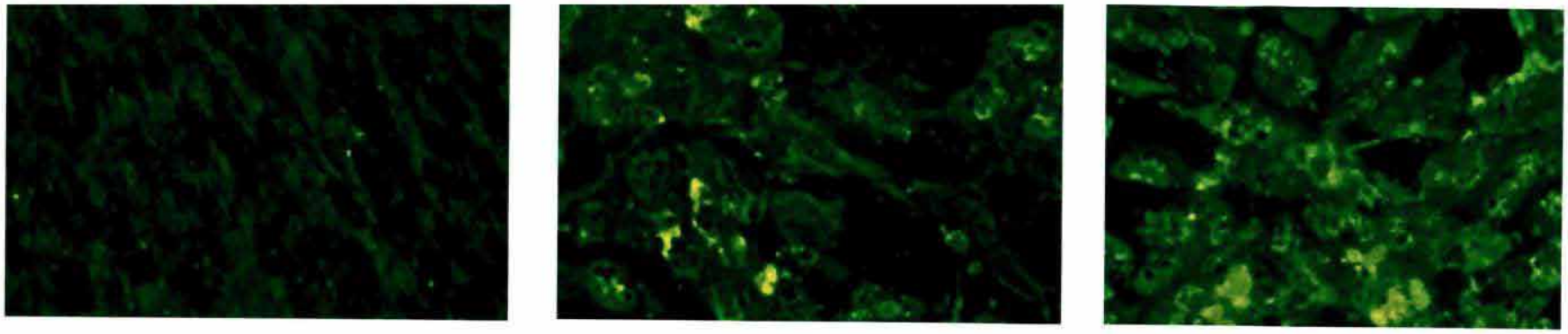
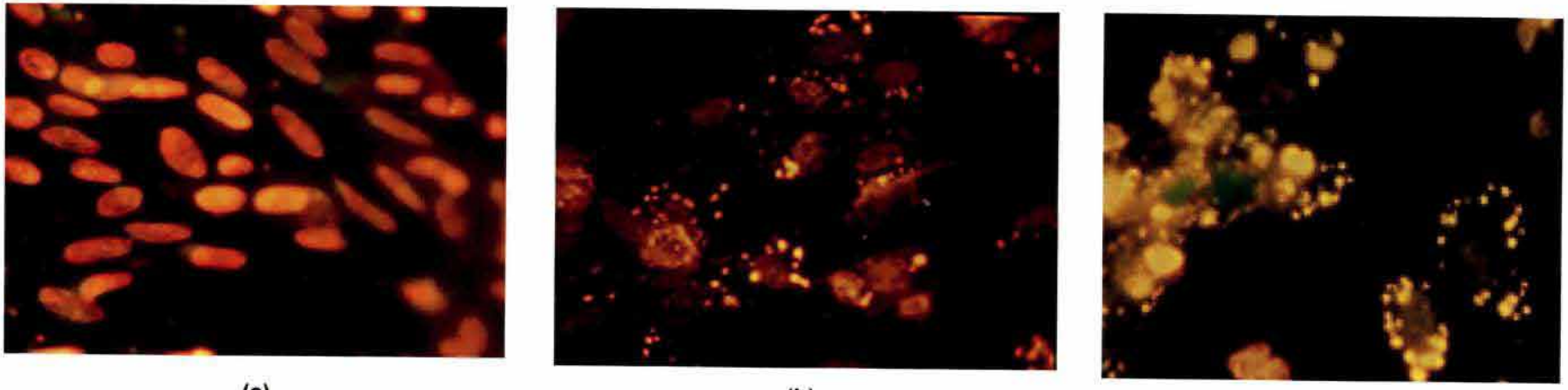


Fig. 33 : RSV transformed CEF cells showing intranuclear immunofluorescence indicating CAV-VP3 expression and apoptotic changes at 48 hr post transfection with pVAX-CAV-VP3. IIFT x 400.



(a) (b) (c)

Fig. 34: Acridine orange-ethidium bromide staining showing apoptic changes viz. nuclear condenstion, fragmentation and formation of small rounded apoptic bodies in RSV transformed pVAX-CAV-VP3 transfected CEF cells. (a) Control; (b) and (c) VP3 transfected. x 400.

Analysis of tumor tissues collected from chicks of group 2 by FAT revealed expression of apoptin in the cells of the tumors (Fig. 36). Analysis of the tissue using acridine orange - ethidium bromide staining also revealed the characteristic sequential changes of apoptosis in nuclear material viz., condensation, fragmentation and formation of apoptic bodies (Fig. 37) in the chicks of Group 2. No nuclear damage could be identified in the chicks of Group 1, 3 and 4. Histopathological analysis of the tumor tissue of group 2 showed severe progressive changes of cell death due to apoptosis. The cells showed condensation of nuclei, fragmentation of chromatin and formation of apoptic bodies (Fig. 38). The apoptotic changes were progressive towards the healthy portion of the tumor tissue and the cells, which had undergone apoptosis, showed loss of nuclear material and dissolution of cell structure, which were seen adjacent to the normal cells in the tumor tissue sections. In between the healthy cells and degenerated cells with dissolved nuclei, a layer of cells showing all characteristic changes of apoptosis was observed. It indicated that the apoptosis was progressive towards the healthy tumor cells.

Tumor: body weight ratio of groups 1 and 2 were 4.97±3.29 and 3.26±2.09, respectively, whereas in groups 3 and 4 were 5.92±4.55 and 2.79±2.12, respectively (Table 13). It indicated that the groups did not differ significantly in the level of tumor suppression but apoptin injected groups (gp 2 and 4) showed lower value than the other groups (gp 1 and 3). The *in vivo* study on anti-neoplastic effect showed that the apoptin suppressed the tumor to some extent by inducing apoptosis. The *in vitro* observations also revealed that apoptin induced apoptosis in RSV transformed chicken embryo fibroblast cells.

Fig. 35a: Development of tumor in RSV inoculated SPF chicks at different development stages (a, b and c) and injected with control plasmid pVAX without any insert.

Fig. 35b: The site of pVAX-CAV-VP3 injection showing contraction, formation of scar tissue and suppression of tumor at different stages. (a, b and c).

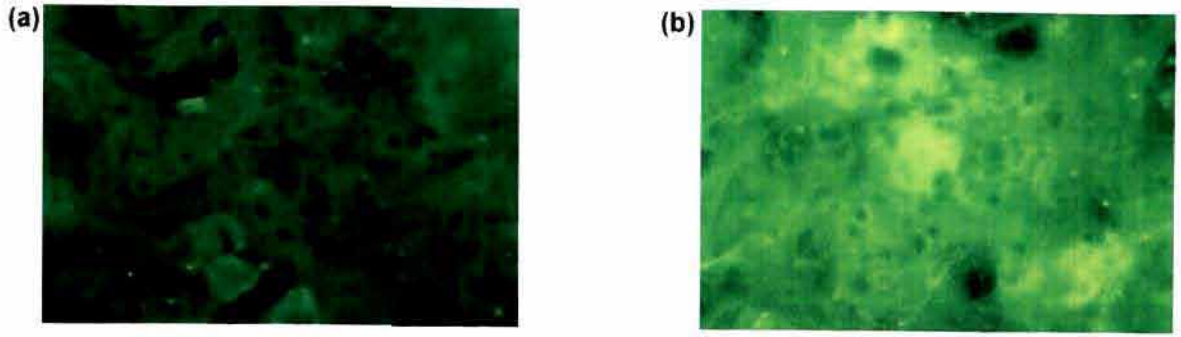


Fig. 36: Tumor tissue injected with pVAX-CAV-VP3 showing expression of apoptin at 5 days post inoculation (a-control; b-VP3 inoculated).

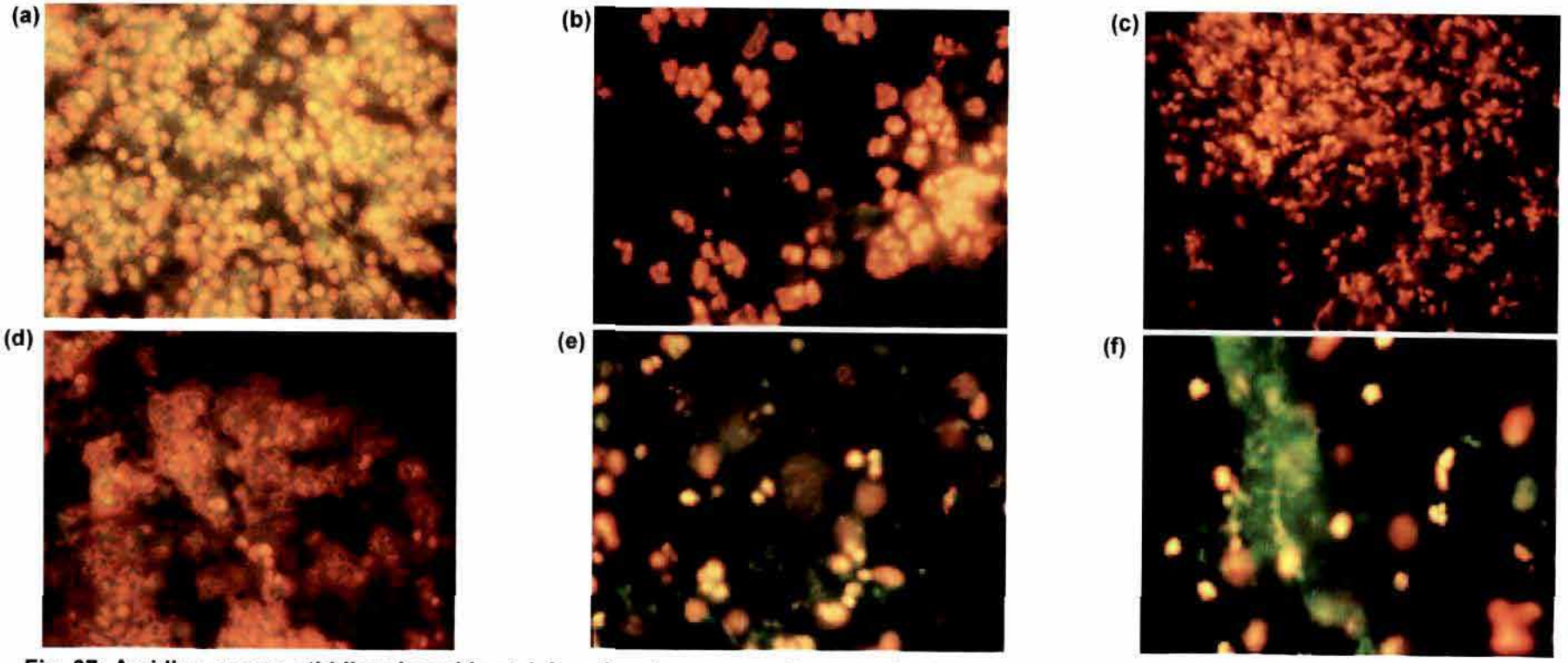


Fig. 37: Acridine orange-ethidium bromide staining showing progressive apoptic changes of nuclear condenstion, fragmentation and formation of small rounded apoptic bodies at different stages in RSV induced tumor injected with pVAX-CAV-VP3 (a- Control; b, c, d, e and f VP3 iniected).

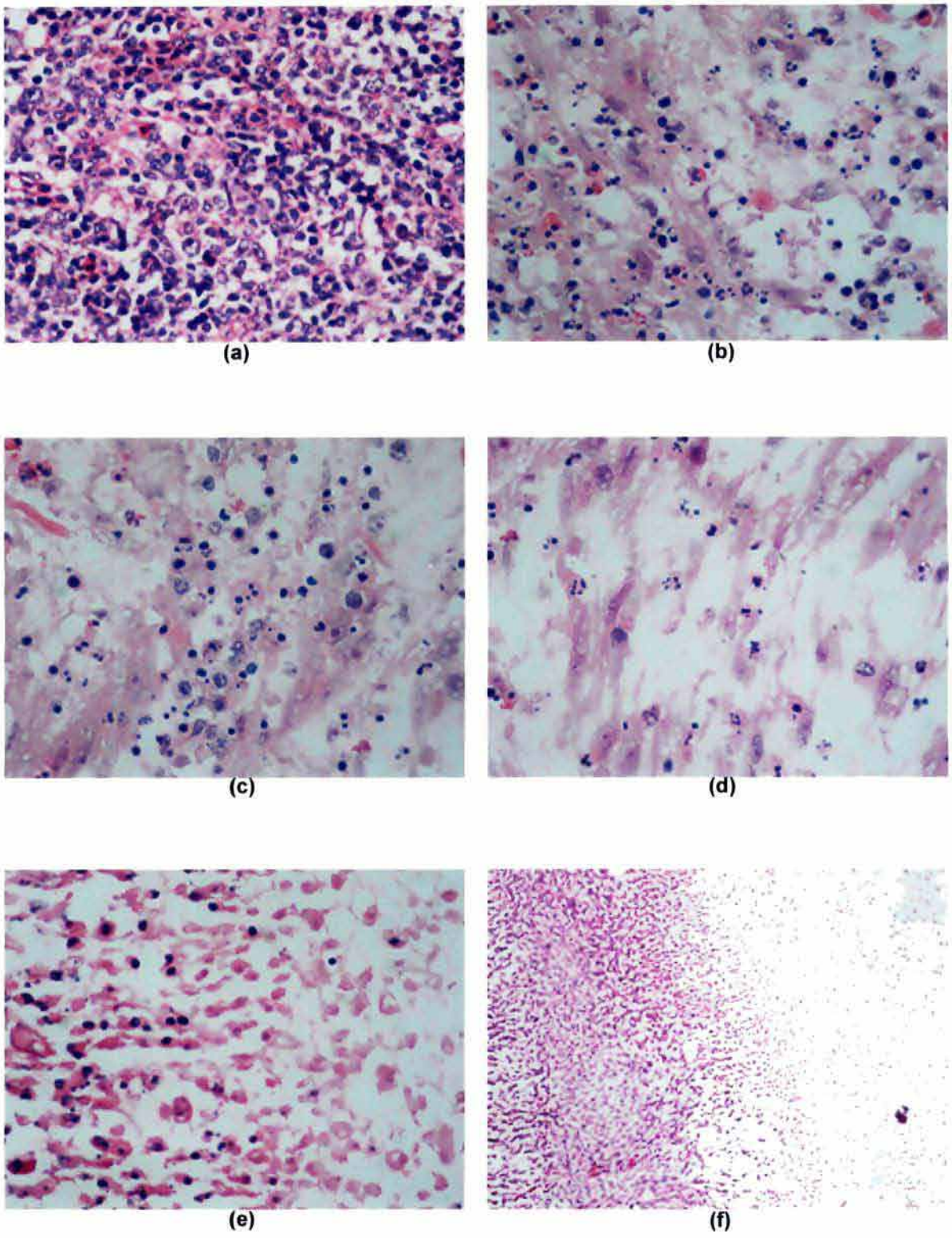


Fig. 38: Histopathological examination using H & E staining showing apoptic changes of nuclear condenstion, fragmentation and formation of small rounded apoptic bodies at different stages in RSV induced tumor inoculated with pVAX-CAV-VP3 (a- Control; b, c, d-different stages of apoptosis progression, e-showing partially cells undergoing apoptosis with some cells without nucleus (H & E x 400), f- similar to e at H & E x 200.

Table 13. Body weight, tumor weight & tumor: body weight of the different groups studied for the anti-neoplastic effect of CAV-VP3 (apoptin).

Observations	Group 1	Group 2	Group 3	Group 4
Body weight (Mean±SD) N=10	122.6±26.32	124.00±6.046	227.57±40.01	212.50±20.99
Tumor weight (Mean±SD) N=10	6.05±4.47	3.96±2.47	6.95±5.68	2.89±2.19
Tumor: B. Wt (Mean±SD) N=10	4.97±3.29	3.26±2.09	5.92±4.55	2.79±2.12
Mortality	2	1	3	0
DNA laddering				
FAT staining	0/7	3/9	ND	ND
Double staining	0/7	4/9	ND	ND

ND-Not detected. Tumor: Body Wt.= (Tumor Wt / Body Wt) X 100

DISCUSSION

Chicken anaemia virus (CAV), etiological agent of a highly immunosuppressive disease of young chicken i.e. chicken infectious anaemia (CIA), is an economically important avian pathogen worldwide. It belongs to *Gyrovirus* genus of the *Circoviridae* family having circular single stranded DNA genome of 2.3 kb in size. The disease is characterized by severe anaemia, aplasia of bone marrow and generalized lymphoid atrophy with concomitant immunosuppression. Immunosuppression is the inability of a host self defense mechanisms against any pathogen. When the self-defense system is damaged by CAV, the host susceptibility increases to all the pathogens of bacterial, viral and fungal origin, which ultimately leads to severe disease outbreaks that are obviously multifactorial. As far as chicken is concerned, infectious bursal disease virus causes severe humoral immune response suppression whereas CAV causes severe cell mediated immune response. Obviously any effect on one also affects the other and the effects can be synergistic leading to increase in severity of disease conditions and huge economic loss to the poultry industry worldwide. In India, the disease was reported in 1994 by Venugopalan and coworkers and thereafter not much information was available on CAVs circulating in the poultry population of the country. Later, Kataria *et al.* (1999) confirmed the wide prevalence of this virus from various states of this country and Dhama (2002) found some variation among the Indian isolates based on RE analysis of the PCR products. Therefore, need was felt for further molecular and pathogenic characterization of these viruses to find out the variation among them, if any, which would help devising suitable control strategy to prevent losses in the poultry industry due to CAV. In this new millennium, sequencing of the complete human genome is one of the greatest achievements in science. Also, sequencing was chosen as a tool to study the molecular epidemiology of Indian CAV isolates in the present study. The usefulness of RE analysis to differentiate among the Indian isolates has also been studied, which will obviously help to the less equipped laboratories.

A nucleotide sequence portion of 1766 bp from position 386 to 2151 as per Meehan *et al.* (1992) in the genome of CAV was sequenced in the present study. The sequences obtained for the four Indian CAV isolates (CAV-A, CAV-B, CAV-E

and CAV-P) were aligned with several CAV isolates of different countries. This region comprised of the complete nucleotide sequences of VP2 and partial sequence of VP1 proteins of CAV. Analysis indicated variation among the Indian isolates ranging from 12 to 52 nucleotides (0.5 to 4.0%). The CAV-E showed maximum variation of 4% among the Indian strains; however, it showed maximum identity of 99.3% with Australian 704 strain. It also showed more than 99% identity with Japanese TR-20 and Malaysian SMSC-1 strains. CAV-B and CAV-P showed 12 and 15 nucleotides variation, respectively among the Indian isolate but had more than 99% identity with Bangladesh BD-3 strain. CAV-A had 13 nucleotides variation and showed 99.8% identity to European Cux-1 strain.

The analysis of 431 amino acid sequences of the VP1 region among the Indian strains indicated 5-7 amino acids variation (0.7 to 2.1%). A maximum variation of 7 amino acids was observed in CAV-P and amino acids at positions 144 (H), 262 (D) and 275 (F) were found specific to the strain. CAV-A, B and E showed only 5, 6 and 5 amino acids variation, respectively. It has been found that CAV-E had 100% identity to Australian 704 strain and CAV-B had 100% identity to Bangladesh BD-3 strain and CAV-A showed 99.5% identity to the European strain Cux-1.

Similarly, Pallister *et al.* (1994) sequenced the PCR product of an Australian isolate and compared the amino acid sequence of VP1 region with German and American isolate and reported 73 nucleotide difference between Australian and German sequences and 80 nucleotide difference between Australian and American sequence. These differences resulted to 12 amino acid changes in a total 449 amino acid, 8 of which were consecutive. Brown *et al.* (2000) sequenced a pathogenic Australian isolate and reported an overall nucleotide sequence identity of approximately 95% indicating close relationship. Farkas *et al.* (1996) determined the complete nucleotide sequence of a CAV strain and concluded that N-terminal half of VP3 and N-terminal three quarter of VP2 are well conserved and might sustain an essential function of these proteins. They also suggested that amino acid changes in VP1 might influence the antigenic variations among the CAV isolates. Kato *et al.* (1995) sequenced a Japanese isolate CAA82-2 and found 98% identity with European Cux-1 strain. A low level of (~3-7%) sequence diversity was identified among the CAV isolates, which was in consistent to finding that only one serotype existed worldwide (Dhama *et al.*, 2002). Recently, Spackman *et al.* (2002a. 2002b)

have reported an antigenically different virus viz., CAV-7 from the American reference CAV strain - Del Ross and suggested that it could be a prototype virus of serotype-2. This report emphasized the need of reviewing the epidemiological status of CAV isolates worldwide.

Renshaw *et al.* (1996) reported a hypervariable region spanning 13 amino acids in VP1 region from position 139 to 151. Naturally occurring amino acid changes at position 139 and 144 has been shown to markedly affect the growth and spread of CAV isolates in cultured cells. In culture, it has been proven that VP1 Q-139 and /or Q144 is associated with decreased rate of spread. Also, it is unclear which of the five amino acids upstream of the hypervariable region (VP1, N-23, I-75, Y-93 and L-07; VP2, E-175) are involved in the block of certain CAV permissible cell lines. The Indian isolates CAV-E and CAV-B had Q-139 and Q-144 indicating that they might spread slowly in MSB-1 cells, however, they were adapted in cell culture by giving more number of blind passages. CAV-A had K-139 and D-144 and CAV-P had Q-139 and H-144. The CAV-P was very well adapted in cell culture. The present study indicated that isolate having Q-139 and H-144 is highly permissive to MSB-1 cells. Brown *et al.* (2000) observed a cluster of non-synonymous changes among the CAV isolates at the hypervariable region and suggested to have biological significance reflecting the selection of variants in response to environment/host cell changes or immune selection. They found remarkable variability at position 144. The present study also supports the finding of maximum variability at position 144 among the Indian isolates.

Yamaguchi *et al.* (2001) reported that amino acid at position 394 in VP1 could be a major genetic determinant of virulence. If it is glutamine (Q) at this position, the isolates are highly pathogenic and if it is histidine (H) than less pathogenic. All the Indian isolates had glutamine (Q) at this position indicating that all are highly pathogenic. This finding has also confirmed the results of pathogenicity study of these isolates in day old SPF chicks as has been discussed in the later part.

The comparative pathogenicity study of all the five CAV isolates was carried out in day old SPF chicks inoculated with a constant dose of 1 ml of the cell culture fluid having titre of $10^{4.5}$ $TCID_{50}$/0.1ml. All the isolates produced consistent low PCV values, reduction in body weight, aplasia of bone marrow and generalized lymphoid atrophy and histopathological lesions of lymphocyte degeneration and depletion from

the lymphoid organs. The lesions closely resembled those as has been described by other workers in SPF chicks inoculated with CAV at day old age (Yuasa *et al.*, 1979, Taniguchi *et al.*, 1982, 1983, Dhama, 2002).

Therefore, the present study revealed good correlation between the molecular study as reported by Yamaguchi *et al.* (2001) with respect to the amino acid Q (glutamine) at position 394 and the *in vivo* pathogenicity study in SPF chicks. From the study, it could be concluded that the amino acid at this position (394) should be identified for newly emerging CAV isolates in the field to assess the pathogenicity. The pathogenicity study could be conducted for further confirmation. This approach will help to identify low pathogenic CAV in the field, if any, and use of it for developing a live vaccine.

RE analysis has been proven useful to differentiate CAV isolates at genome level (Todd *et al., 1992*, Noteborn *et al.*, 1992; Santeen *et al.*, 2001). The present study also revealed that restriction enzymes viz. *Hha* I, *Dde* I, *Sac* I and *Hae* III were useful for the differentiation of the CAV field isolates. *Hha* I differentiated CAV-A, B or P and E isolated by yielding different pattern. *Dde* I enzyme was found to group A and E and B, and P separately, whereas *Sac* I digestion grouped A and P together, and B and E isolates together. Among these enzymes, *Hha* I was found very useful to differentiate more number of isolates. *Hae* III was also found useful to differentiate CAVs using both VP1 and VP2 regions. Noteborn et al. (1992) used the enzymes viz., *Eco* RI, *Acc* I, *Bgl* II, *Hind* III, *Sst* I, *Bam* HI, *Xba* I and detected variation among the isolates by *Acc* I, *Hind* III and *Eco* RI enzymes. Similarly, Todd *et al.* (1992) analyzed 14 CAV isolates obtained different parts of the world and assigned them to 7 groups based on the restriction pattern of the PCR amplified 675 bp region of N- terminal half of VP1 using the enzymes viz. *Hae* III, *Hinf* I and *Hpa* I. The present study also indicated the usefulness of restriction enzymes viz. *Hha* I, *Dde* I, *Sac* I and *Hae* III to differentiate the Indian field isolates which would be helpful for the less equipped diagnostic laboratories.

Nucleic acid immunization is a novel method for the production of third generation vaccines. Many reviews on nucleic acid immunization have been published in the recent past (Oshop *et al.*, 2002). It is a novel approach to achieve both cell mediated as well as humoral immune response. Nucleic acid immunization has several advantages over the conventional vaccines i.e. a) they mimic the effects

live virus in their ability to induce major histocompatibility complex (MHC) class I restricted CD8+ T-cell responses without any concern about virulence b)easy to design and manufacture in relatively cost effective manner c) easy to store eliminating the need of cold chain d) overcome the inherent unresponsiveness in neonatal animals and e) can work in the face of maternally derived immunity. Many vaccines have been developed for the use in poultry against various diseases viz. IBD (Foddor *et al.*, 1999; Chang *et al.*, 2001), influenza (Fynan *et al.*, 1993; Robinson *et al.*, 1993; Kodihalli *et al.*, 1997; Kodihalli *et al.*, 2000; Suarez and Schultzcherry, 2000) duck hepatitis-B (Triyatni *et al.*, 1998; Rollier *et al.*, 1999; 2000a; 2000b). Therefore, in the present study, nucleic acid immunization approach was used for the immunogenic characterization of the CAV-VP1 and VP2 proteins. It has been reported that CAV-VP1 and VP2 are required to achieve higher level of protection against the disease (Koch *et al.*, 1995; Noteborn *et al.*, 1998). Koch *et al.* (1995) studied the immunogenic properties of the CAV proteins (VP1, VP2 and VP3) using baculovirus vector and expressed in insect cells. The proteins produced separately or together in insect cells cultures were analyzed by inoculating into the chickens. It was found that, only lysates of insect cells, which have synthesized equal amount of all the three recombinant CAV proteins, or cells which synthesized mainly VP1 plus VP2, induced neutralizing antibodies directed against CAV in inoculated chickens. Progeny of these chickens were protected against clinical disease after CAV challenge. Noteborn *et al.* (1998) reported that CAV VP1 and VP2 interaction is essential for the formation of neutralizing epitope. Cunningham *et al.* (2001) reported that CAV-VP3 protein was poorly immunogenic as well as poorly antigenic and not useful for developing ELISA. Keeping the above reports in mind, the CAV VP1 and VP2 were used combinedly for the nucleic acid immunization approach to achieve the maximum protection in the present study. The CAV-VP1 and VP2 were cloned into eukaryotic expression vector pVAX and their *in vitro* expression was studied. CAV-VP1 was expressed to high level in Hep-2 cells, which was demonstrated by employing indirect immunofluorescent technique using CAV specific neutralizing monoclonal antibodies. The expression of VP2 was demonstrated in CEF cells using the CAV specific polyclonal antiserum.

The study was aimed to attain high level of protection to the newly hatched chicks either through the maternal antibody from the immunized adult birds or directly

via *in ovo* immunization. The study was conducted in 18 weeks old laying hens along with cocks, divided into 3 groups. Each group comprised of 10 birds, 8 of which were hens and 2 were cocks. Group 1 was kept as control. Group 2 was injected with single dose of vaccine having 100 μg of pVAX-CA-VP1 and 100 μg of pVAX-CA-VP2 combinedly. Group 3 received a second (booster) dose of same amount of plasmid as given earlier, on 15th day.

Kapczynski *et al.* (2003) developed a DNA vaccine using S1 glycoprotein against the infectious bronchitis virus. They used 50, 100 or 150 μg of DNA vaccine at day 1 and 100, 200 or 300 μg of DNA vaccine, respectively at 14 days of age and found that no clinical signs were observed and no challenge virus was reisolated from the birds vaccinated with 150 and 300 μg of DNA injected intramuscularly. Babuick *et al.* (2000) developed a DNA vaccine against avian influenza and found immune response induced by the vaccine was dose dependent. A dose of 100 μg/bird was found to give maximum response over which a decrease in immune response was reported in young chicks. Hence, in the adult layer birds, a dose of 200 μg/bird was used for immunization in the present study. However, a dose rate ranging from 5 μg to 800 μg has been tried in chicken through various route of inoculation for the nucleic acid immunization. In general, very low level of plasmid (0.25-10 μg) was used when injected intra-dermaly using gene gun. In the present study, the plasmid was injected intramuscularly in thigh region which is the most common route tried by many workers (Oshop *et al.*, 2002).

In the present study, the cell mediated as well as humoral immune response in the immunized birds was measured at weekly interval. The cell mediated immune response in vaccinated layer birds was assayed by measuring stimulation indices (SI) of peripheral blood mononuclear cells of the immunized chicks employing LTT using MTT dye following the method of Bonous *et al.* (1992). Group-2 showed gradual increase upto 14 days post immunization (1.424±0.056) and it showed declining trend from 21 days (1.311±0.056) onwards. In group-3, the CMI response increased gradually upto 28 days post immunization (1.600±0.062). Gurunathan *et al.* (2000) reported that nucleic acid immunization induced humoral and cell mediated immunity through the priming of CD4+ and CD8+ cells. The CMI response plays major role in the protection of chickens against the CAV infection. Seo *et al.* (1997)

reported that the carboxyl terminal 120 residue polypeptide of infectious bronchitis virus nucleocapsid protein induced cytotoxic T-lymphocytes and protected young chicks from acute infection. Vanrompay *et al.* (1999) also reported that a DNA vaccine using the major outer membrane protein (MOMP) of the chlamydia induced CMI response in turkeys.

The humoral immune response in the immunized birds was measured using the CAV specific ELISA. Group 2 showed maximum antibody titre of 4033±76.97 on 21st day and the antibody titre started declining and was 3811.63±52.04 on 28th day whereas group 3 showed gradual increase throughout the observation period with maximum titre of 6538.50±86.64 on 28th day. The antibody titre of the group 2 increased gradually upto 21 DPI and started decreasing on 28 DPI. It indicated that the antibody response due to single dose immunization is lasting for short duration. However, the group 3 showed a gradual increase upto the observation period of 28 DPI. Obviously, the booster dose increased the antibody titre to a significantly higher level. The reason for the high level of cell mediated and humoral immune response in the group 3 might be due to the presence of memory cells created by the initial dose of immunization.

Only in ducks, Rollier *et al.* (2000a) have reported that immunization of the adult bird using the L protein of duck hepatitis-B virus protected the chicks. They have also demonstrated presence of IgY in yolks. Also, in the present study, it was found that the adult birds immunized with CAV-VP1 and VP2 conferred complete protection from the clinical disease and partial protection from the immunosuppression to their young chicks, which was indicated by the presence of microscopical changes in 2 of the 10 immunized chicks in group 2. A total 8 out of 10 birds have shown complete protection from the CAV induced immunosuppression since no microscopic changes of lymphocyte depletion were seen in lymphoid organs. The available literatures on DNA vaccine also indicate that the protection is mostly partial even after repeating the dose twice or thrice (Fynan *et al.*, 1993a; Robinson *et al.* , 1993; Sakaguchi *et al.* , 1996; Cheng *et al.*, 2000; Triyatni *et al.*, 1998). Suarez and Schultzcherry (2000) reported that DNA vaccine using the HA gene of A5 type gave complete protection in birds boosted at 3rd weeks. Keeler (2000) also reported 80-100% protection in chickens immunized with GB protein gene of ILT virus.

Inoculation of DNA vaccines through the *in ovo* route has been tried by few workers (Oshop *et al.*, 2002). Recently, Kapczynski *et al.* (2001) reported that birds receiving *in ovo* DNA vaccine using infectious bronchitis virus S-1 glycoprotein gene followed by live vaccination at 2 week of age were completely (100%) protected from clinical disease. Also, birds receiving only live virus vaccine or only *in ovo* DNA vaccination were ≤ 80% protected. In the present study, *in ovo* immunization was studied to evaluate the nucleic acid immunization for achieving the protection against CAV in newly hatched chicks. The antibody titre was measured at weekly interval by ELISA. All the chicks were challenged at 15th day of age. The antibody titres following challenge virus inoculation were significantly higher in the *in ovo* immunized chicks with the titres of 3081.00±122.78 and 4482.80±124.30 than the control chicks titre of 1516.80±167.31 and 3202.10±111.74 on 21 and 28 days of age, respectively. The chicks were also protected completely against the clinical disease but partially against the virus-induced immunosuppression. The reason for the higher level of antibody development to the infection in *in ovo* vaccinated chicks might be due to the presence of memory cells, which might have been sensitized by the inoculated DNA. However, the study revealed that *in ovo* immunization could be a novel method for the control of CAV infection at the most susceptible age of less than 3 weeks. It can be combined with a live virus vaccine at 2 weeks of age as reported by Kapczynski *et al.* (2003) to achieve the complete protection against the immunosuppression.

CAV-VP3 is a very small protein of 13kDa in size, which is produced abundently during the virus replication in the host cells and it is poorly antigenic in nature (Cunningham et al., 2001). The apoptosis inducing property of chicken anaemia virus was demonstrated by electron microscopy and biochemical methods in cortical thymocytes after *in vivo* infection and in lymphoblastoid cell line after *in vitro* infection (Jeurissen *et al.* 1992). Later, Noteborn *et al.* (1994) reported that chicken anaemia virus VP3 protein alone induced apoptosis in chicken mononuclear cells and lymphoblastoid cell line. Following these reports, the research work was focused to exploit it's apoptotic potential against the neoplastic cells.

Zhuang *et al.* (1995) reported apoptosis induced by apoptin was independent of p53 protein and Pieterson and Noteborn (2000) suggested apoptin could be a candidate for treating the tumor cells resistant to many chemotherapeutic agents which normally exhibit the action via P^{53} protein - a key protein that induce apoptosis

in cells. Danen van Oorschot *et al.* (2003) have reported CAV-VP3 is expressed to a high level in transformed cells and to a very low level in normal cells and also reported that apoptin predominantly localized in the nucleus of the transformed cell and in the cytoplasm of the normal cell.

However, the effect of this protein has not been tested *in vivo* against any of the virus-induced tumors. Thus, the present study was undertaken to observe the effect of apoptin in RSV induced tumors. The objective was whether the apoptin have some tumor suppressive effect in RSV induced tumor. In the event of tumor suppression, attempts were made to confirm whether it is due to apoptosis induced by apoptin. The results of the present study indicated that the tumor suppression was due to apoptin-induced apoptosis, which was confirmed by *in vitro* and *in vivo* studies. When the apoptin was injected intra-tumorally, tumor suppression was observed grossly at the site of inoculation. Further, it could be confirmed that tumor suppression was due to apoptin expression in the tumor cells and induction of apoptosis by employing fluorescent antibody technique, acridine orange - ethidium bromide staining and histopathology. *In vitro*, the induction of apoptosis by the apoptin in RSV transformed CEF cells was also confirmed by analyzing the cells for the cytomorphological changes by fluorescent antibody technique and acridine orange-ethidium bromide staining. In these cases, the characteristic changes of nuclear condensation, fragmentation of chromatin and formation of small rounded apoptotic bodies in the cells undergoing apoptosis were seen. The tumor suppression was noticed only at the site of injection. The suppression level was not significantly different from the control group as determined by tumor: body weight in the present study. The reason could be a very fast growth of RSV induced tumor than the rate of apoptin induced cell death. The dose of apoptin, number of injection, site of injection could also influence the efficiency of tumor suppression by apoptin.

Recent reports of LeLiveld *et al.* (2003a and 2003b) and Danen van Oorschot *et al.* (2003) explained the mechanism of apoptin-induced apoptosis in tumor cells to some extent. Still, the complete mechanism is unclear. LeLiveld *et al.* (2003b) reported that apoptin predominantly co-localized with heterochromatin and nucleoli within tumor cells on the basis of immunoelectronmicroscopy. They have also reported that apoptin co-operatively forms distinct superstructures with DNA *in vitro* and these superstructures do not go beyond 20 multimeric apoptin complexes and

approximately 3 Kb of DNA. Further, they showed that a single apoptin multimer to have eight independent non-specific DNA binding sites which predominantly bind strand ends. Apoptin also have high affinity for naked, uncoated double and single stranded DNA. Since this form of DNA are predominantly found in transcriptionally active. Danen van Oorschot *et al.* (2003) explored the role of nuclear localization for apoptin-induced cell death in tumor cells by employing mutagenesis strategy. They demonstrated that C-terminal of the apoptin contains a bipartite-type nuclear localization signal. Further investigation showed that apoptin contains two domains that induce apoptosis independently and for both domains had strong correlation between localization and killing activity. They also reported that *de nova* gene transcription and translation involved in the process of cell death and suggested apoptin exerted its effects in the nucleus by some other method, and concluded that nuclear localization alone is not sufficient for the apoptin to become active and to induce apoptosis and cell death. The present study also clearly showed the localization of apoptin in the nucleus of the virus induced tumor cells and induction of apoptosis but it could not do so in the normal cells. Further information on how the apoptin differentiates the normal cells and specifically recognizes tumor cells, and which mediates the nuclear localization signal will help for the specific targeting of the tumor cells.

The present study revealed the presence of genetically different chicken anaemia viruses circulating in the field of this country. These CAV strains are closely related to European, Australian, Japanese and Bangladesh strains which indicated that Indian CAV isolates might have been originated from different parts of the world. It warrants the need for constant monitoring of this virus in the field for emergence of any new variant virus since there is more chance for that due to the presence of many genetically different viruses circulating in the field. All the five Indian isolates are found pathogenic to young chicks and cause severe immunosuppression. Also, these isolates do not differ significantly in pathogenicity. It could be concluded that the genetic difference did not reflect in their pathogenicity. The combined use of CAV-VP1 and VP2 for the nucleic acid immunization was found useful to protect the newly hatched young chicks either through the maternal immunity conferred by the vaccinated dams or through *in ovo* immunization at embryo stage. It could become an alternative method to develop a third generation vaccine against CAV. However, it

requires further modification to achieve complete protection either by use of adjuvants or inoculation using gene gun intra-dermally or repeated inoculation of the vaccine. *In ovo* nucleic acid immunization combined with a live vaccine at 2nd week is also a possible way to achieve the complete protection. The anti-neoplastic effect of apoptin in virus-induced tumor was confirmed *in vivo* and *in vitro*. The apoptin has potential to be used as an ideal anti-neoplastic drug for treating the virus-induced tumors due to its specific targeting and selective killing. The studies on other aspects viz. dose and number of inoculations, and different routes need to be explored to achieve complete tumor suppression.

SUMMARY

Chicken infectious anaemia (CIA) is a highly immunosuppressive disease of young chicken characterized by severe anaemia, atrophy of thymus and aplasia of bone marrow. The disease is caused by chicken anaemia virus (CAV) belonging to *Gyrovirus* genus of *Circoviridae* family, which has a single stranded circular DNA genome of 2.3 kb. It has three proteins viz. VP1, VP2 and VP3. CAV-VP1 is a major capsid protein of 54 kDa, 441 amino acids; VP2 is a non-structural scaffold protein of 24 kDa, 216 amino acids and a 13 kDa VP3 protein, 121 amino acids called apoptin (CAV-VP3) due to its apoptosis inducing property. The present study was carried out to characterize the CAVs circulating in India at genomic level by nucleotide sequencing and restriction endonuclease analysis in order to find out the epidemiological status of the disease in this subcontinent. A total 5 isolates (CAV-A, CAV-B, CAV-C, CAV-E and CAV-P) obtained from different geographical regions of the country, were sequenced partially. The 1766 bp nucleotides from position 386 to 2151 (as per Meehan *et al.*, 1992) of the four isolates were sequenced. One of the isolates CAV-C was sequenced for 651 bp region of the CAV-VP2. The sequences obtained were edited in EditSeq programme of the Lasergene software and aligned by Clustal method in Megalign programme of the same software along with the sequences of foreign isolates downloaded from the NCBI website. The amino acid sequences were also translated from the nucleotide sequences using EditSeq programme and aligned by Clustal method.

Analysis of the 1766 bp nucleotide sequence of the Indian CAV isolates revealed a variation ranging from 15 to 52 nucleotides among them, which showed 0.5 to 4.0% divergence. Among the Indian and foreign isolates, the variation ranged from 0.2 to 4.1%. The Indian isolates CAV-B and CAV-P had ≥99% identity with Bangladesh isolates BD-3 strain. CAV-A had maximum identity with European Cux-1 isolate. CAV-E was having maximum identity with Australian 704 (99.3%), Japanese TR-20 (99.2%) and Malaysian SMSC-1 (99%).

Deduced 431 amino acid sequence analysis of CAV strains of Indian and foreign origin indicated variation ranging from 0 - 2.8%, CAV-E showed 100% identity with Australian 704 strain. Similarly, CAV-B showed 100% identity with Bangladesh BD-3 strain. CAV-A had 99.5% identity with Cux-1 strain. Deduced amino acid analysis of 216 amino acid sequence of VP2 showed only one amino acid variation at

position 149 of CAV-A and at position 186 of CAV-E. It indicated that CAV-VP2 is highly conserved even among the distant isolates.

Hypervariable region of CAV spanning 13 amino acids sequence is located in VP1 region from position 139 to 150. The region is suggested to have biological significance. Variations at position 139 and 144 were observed among the Indian isolates. CAV-E and CAV-B had Q at position 139 and 144. CAV-A had only one variation at position 144 (D). Similar to Cux-1, CAV-P had Q at 139 and H at 144. It is reported that VP1-Q at 139 and/or Q at 144 is associated with decreased rate of spread of virus in cell culture. The amino acid at position 394 is reported as genetic determinant of CAV pathogenicity. The viruses having Q (glutamine) at this position are considered highly pathogenic and H at this position are low pathogenic. All the Indian isolates were found to have Q at this particular position indicating that all are highly pathogenic.

Phylogenetic analysis based on the nucleotide and amino acid sequences of Indian and foreign CAV strains indicated CAV-A and Cux-1; CAV-B and CAV-P are closely related to the Bangladesh BD-3 strain. CAV-E is closely related to Australian 704, Japanese TR-20 and Malaysian SMSC-1. All Indian isolates are not closely related to American CAV isolates. This study indicated that the Indian isolates are genetically different and might have been originated from different parts of the world viz., Europe, Australia and South East Asia.

The present study also revealed that restriction enzymes viz. *Hha* I, *Dde* I, *Sac* I and *Hae* III were useful for the differentiation of the CAV field isolates using the PCR amplified VP1 regions of CAV. *Hha* I differentiated CAV-A, B or P and E isolated by yielding different pattern. *Dde* I enzyme was found to group A and E and B, and P separately, whereas *Sac* I digestion grouped A and P together, and B and E isolates together. *Hae* III differentiated CAV-E from all other isolates based on the VP1 as well as VP2 regions.

Pathogenicity of all the five Indian isolates was studied in day old SPF chicks. The chicks were inoculated with 1 ml of cell culture fluid having $10^{4.5}$ $TCID_{50}$/0.1ml and clinical signs, mortality, PCV and body weight gain were recorded on 5, 10 and 15 DPI. On 15^{th} day, the chicks were sacrificed and the gross lesions were recorded and tissues were collected for microscopic examination. The study indicated that all the five Indian isolates of CAV are highly pathogenic and no significant variation in the pathogenicity was found among them. The PCV value at 15^{th} DPI of CAV infected chicks of all the isolates ranged from 12.83±3.32 to 15.33±2.07 compared to the

control value of 32.33±3.39 indicating severe anaemia. Similarly, the body weight of the infected chicks was very low ranging from 85.0±05.62 to 89.00±4.90g compared to the control group body weight of 103.50±5.43g on 15th DPI. All the isolates produced atrophy of lymphoid organs and severe depletion of lymphocytes.

Protective effect of the CAV-VP1 and VP2 proteins in layer birds was studied through nucleic acid immunization approach. The CAV-VP1 and VP2 genes were cloned into pVAX expression vector and the expression of the protein was studied in Hep-2 cells and CEF cells, respectively. The expression of these proteins was confirmed by indirect immunofluorescent technique. To study the *in vivo* protective effect in layer birds, thirty birds were divided into 3 groups consisting of 10 in each group. Each group had 8 laying hens and 2 cocks. Group 1 served as control, which received pVAX without any insert. Group 2 and 3 were vaccinated with 200 µg of pVAX (VP1-100 µg + VP2-100 µg) on day 1. Later, on 15th day, group 3 received a booster dose of same amount of plasmid as given on day 1. The birds were monitored for cell mediated immune response (CMI) by LTT assay and humoral immune response (HIR) by CAV specific ELISA at weekly intervals up to 28 days. No difference among the group 2 and 3 was found upto 14 days. The group 3 showed significantly high SI value (1.579±0.096 and 1.600±0.062) than the group 2 (1.311±0.056 and 1.191±0.074) at 21 and 28 days post vaccination, respectively. The humoral immune response was found gradually increasing and maximum ELISA antibody titre was observed on 21 DPI (4033.38±76.97) and thereafter it started decreasing and it was 3811.63±52.04 at 28 DPI in group-2 where as in group 3, the HIR increased gradually and had maximum titre of 6538.50±86.64 at 28 DPI. The fertile eggs from each group were collected between day 21 and 28. The chicks were hatched out from the eggs and challenged with CAV at day old, and observed for the clinical signs, mortality, PCV and body weight on 5, 10, 15 days post infection. At 15th day, all the chicks were sacrificed and gross lesions and microscopic changes were recorded.

The study revealed that the layer birds, which received 2 doses of vaccine developed high level of CMI and HIR, and the young ones hatched from them were completely protected against the clinical disease and partially protected from the *virus* induced immunosuppression as compared to the control and single vaccinated groups.

The anti-neoplastic effect of CAV-VP3 (apoptin) was studied in Rous sarcoma virus transformed CEF cells *in vitro* and in RSV induced tumors of SPF chicks *in vivo*. *In vitro* study was aimed to confirm whether the VP3 induces apoptosis in the virus transformed cells and the *in vivo* study was aimed to study (i) whether it suppresses the RSV induced tumor when injected intra-tumorally and (ii) whether it protects the chick from the tumor development when the bird was previously sensitized/treated with apoptin. For this, 4 groups of SPF chicks, each comprising of 10 chicks were used. Group 1 and 2 were injected with RSV on day old age. Group 1 served as control receiving pVAX vector without any insert and group 2 received pVAX-CAV-VP3 at a dose rate of 100 µg per 0.2 ml per bird intra-tumorally. Group 3 received 100 µg/0.2 ml/bird of pVAX vector at day old age and group 4 received the same dose of pVAX-CAV-VP3 at day old age. The group 3 and group 4 were infected with RSV on day 10 post plasmid inoculation. The *in vitro* study confirmed that VP3 induced apoptosis in virus transformed CEF cells, which was confirmed by demonstrating the characteristic changes of apoptosis by fluorescent antibody technique and acridine orange - ethidium bromide staining. The *in vivo* study also indicated that the CAV-VP3 induced apoptosis and caused tumor regression at the site of injection in the tumor. The apoptotic changes viz., nuclear condensation, fragmentation of the chromatin and formation of apoptic bodies in the tumor cells were conformed by histopathology and acridine orange - ethidium bromide staining. No such apoptotic changes were seen in tumors of group 1, 3 and 4. The study revealed that CAV-VP3 could be a useful candidate for treating the retrovirus-induced tumors in particular and virus induced tumors in general. From the findings of present study, following conclusions could be derived.

1. Difference at genomic level was detected among the various CAV isolates circulating in the poultry population of this country.
2. Indian strain CAV-A is closely related to European strain whereas CAV-E is closely related to Australian, Malaysian and Japanese strains. CAV-B and CAV-P are closely related to Bangladesh strain.
3. CAV-E and CAV-B isolates found to have Q (glutamine) at position 139 and 144 in the hypervariable region of VP1, which are responsible for low spreading nature in the cell culture.
4. All the Indian isolates were found to have Q at position 394 which indicated their highly pathogenic nature.

5. Restriction endonuclease analysis revealed that restriction enzymes *Hha* I, *Hae* III, *Dde* I and *Sac* I were found useful to differentiate Indian CAV isolates using the PCR amplified complete VP1 region.
6. Pathogenicity studies in SPF chicks indicated that all the five Indian CAV isolates were pathogenic to young chicks and no significant variation among them was observed.
7. The combined use of CAV-VP1 and VP2 through nucleic acid immunization approach was found suitable method to develop a third generation vaccine against CAV.
8. pVAX vector was found suitable to develop a nucleic acid immunization method for CAV.
9. The CAV-VP1 and VP2 were found to induce cell mediated as well as humoral immune response.
10. Apoptin was found to express in the neoplastic cell line, Hep-2 and induced apoptosis.
11. CAV VP3 (apoptin) apoptotic potential was determined in RSV transformed chicken embryo fibroblast cells *in vitro* and in RSV induced tumors of chicken *in vivo*.
12. CAV-VP3 was found to suppress of the RSV induced tumor by causing apoptosis and further studies are required to exploit its therapeutic potential for virus-induced tumors.

MINI ABSTRACT

The chicken anaemia virus isolates obtained from various parts of India, were characterized at the genomic level. The immunogenic effect of the CAV-VP1 and VP2 proteins and anti-neoplastic effect of CAV-VP3 (apoptin) were also studied. A total of 5 CAV isolates were sequenced partially. The sequence analysis of 1766 bp nucleotides showed variation of 12 to 52 nucleotides among the Indian isolates. CAV-E showed maximum variation of 52 nucleotides. These 52 nucleotides variation (4%) resulted in only 5 amino acid changes. There was only one amino acid variation at position 149 in CAV-A and one at position 186 in CAV-E in the VP2 region. CAV-E showed maximum 4% variation. In VP1 region, variation of 7 amino acids was found in CAV-P, which showed 2.1% divergence. Phylogenetic analysis based on the nucleotide and amino acid sequences of VP1 protein revealed that CAV-A was very closely related to European Cux-1 strain, CAV-E was very closely related to Australian, Malaysian and Japanese strains whereas CAV-B and P were closely related to the Bangladesh strain. RE analysis revealed that restriction enzymes *Hha* I, *Hae* III, *Dde* I and *Sac* I were found useful to differentiate Indian CAV isolates. Pathogenicity study of five Indian CAV isolates in day old SPF chicks indicated that all are highly pathogenic to young chicks. The CAV-VP1 and VP2 genes were cloned into pVAX expression vector and *in vitro* expression of the proteins was studied in Hep-2 and CEF cells, respectively. The immunogenic effect of CAV-VP1 and VP2 protein was studied in 18 weeks old layer birds and in young chicks through the nucleic acid immunization approach. The results indicated that the dams vaccinated with 2 doses conferred complete protection to the young ones from the clinical disease and partial protection from the virus induced immunosuppression. In ovo immunization in 18 days old embryos also conferred protection to young chicks from the clinical disease to CAV challenge at 15th day of age. The CAV-VP3 gene was cloned in pVAX vector and *in vitro* expression of the protein was confirmed in Hep-2 cells. The anti-neoplastic effect of CAV-VP3 protein in RSV transformed CEF cells *in vitro* and in RSV induced tumor of young chicks *in vivo* revealed induction of apoptosis and causing suppression of tumor at the site of injection. The present studies indicated that CAV isolates circulating in this country are genetically different. Indian CAV strains might have evolved from different parts of the world. Further studies on complete genome sequencing of recent field isolates will give further information on epidemiological status of this virus in the country. Nucleic acid immunization with VP1 and VP2 combinedly might be a useful alternative immunoprophylactic against CAV. Further work using adjuvant/immunostimulants is needed to achieve complete protection from the virus induced immunosuppression. The CAV-VP3 may be exploited to use as anti-neoplastic drug for retrovirus-induced tumors.

सारांश

प्रस्तुत शोध में भारत के विभिन्न क्षेत्रों से विलगित किए गये कुक्कुट रक्ताल्पता विषाणु (सीएवी) के वियुक्तों का संजीन स्तर पर अभिलक्षणन किया गया है। इन वियुक्तों के वीपी–1 एवं वीपी–2 प्रोटीन्स के प्रतिरक्षाजनी प्रभाव तथा वीपी–3 (एपोप्टीन) प्रोटीन के प्रति–अर्बुदीय प्रभाव का अध्ययन किया गया है। कुल 5 सीएवी वियुक्तों का आंशिक अनुकम भी किया गया। भारतीय वियुक्तों में 1766 बीपी न्यूक्लिओटाइड के अनुकम विश्लेषण के पश्चात 12 से 52 न्यूक्लिओटाइड में विविधता पाई गई जिनमें सीएवी–ई में सबसे ज्यादा 52 न्यूक्लिओटाइड की विविधता केवल 5 अमीनों अम्ल में पाई गई। सीएवी–ए के वीपी–2 क्षेत्र में सिर्फ एक परिवर्तन जो कि 149 स्थान पर तथा सीएवी–ई के वीपी–2 क्षेत्र के 186 स्थान पर पाया गया। सीएवी–ई में अधिकतम विविधता 4 प्रतिशत की पाई गई। सीएवी–पी के वीपी–1 क्षेत्र में 7 अमीनों अम्ल की विविधता जोकि 2.1 प्रतिशत तिक पाई गई। वीपी–1 प्रोटीन की जातिवृत्तीय विश्लेषण से पता चला कि सीएवी–ए, यूरोपियन कक्स–1 प्रजाति के बहुत निकट सम्बन्धित है तथा सीएवी–ई आस्ट्रेलिया मलेशियन तथा जापानीज प्रजाति के करीब जबकि सीएवी–बी तथा सीएवी–पी बांग्लादेश के प्रजाति के करीब पाया गया।

सीएवी वियुक्तों के रोगजनकता का अध्ययन एक दिन के एसपीएफ चूजों में करने पर सभी वियुक्त अतितीव्र रोगजनक पाये गये। सभी सीएवी के वीपी–1 एवं वीपी–2 जीन को पी. वैक्स रोगवाहक एवं पात्रे में भावाकृत करके हेप–2 एवं सीईएफ कोशिका में अध्ययन किया गया। वीपी–1 एवं वीपी–2 प्रोटीन की प्रतिरक्षाजनी प्रभाव का अध्ययन 18 सप्ताह के अंडे देने वाली मुर्गियों में तथा नौजवान चूजों में न्यक्लिक अम्ल प्रतिरक्षीकरण विधि द्वारा किया गया जिसका परिणाम चूजों में रोग लक्षण से पूर्ण सुरक्षा तथा विषाणु जनित प्रतिरक्षालुप्तांगता आंशिक रूप में पाई गई। 18 दिन के भ्रूणों में प्रतिरक्षणन करने से नौजवान चूजे भी सीएवी चैलेन्ज से पूर्णतया सुरक्षित रहे। सीएवी वीपी–3 जीन की क्लोनिंग हेप–2 कोशिका के माध्यम से सत्यापित की गई।

बीपी–3 प्रोटीन की प्रति अर्बुदीय प्रभाव का अध्ययन आरएसवी रूपान्तरित सीईएफ कोशिका में तथा चूजों में उत्पन्न की गई अर्बुद में एपोप्टोसिस तथा अर्बुद लुप्तांगता द्वारा पुष्टि की गई।

प्रस्तुत शोध से यह ज्ञात हुआ कि हमारे देश में व्याप्त सीएवी वियुक्त आनुवांशिक तौर पर दुनिया के अन्य स्थानों से प्राप्त सीएवी से भिन्न है। अतः आगामी अध्ययन से हमारे देश के विभिन्न क्षेत्रों से हाल में विलगित किये गये सीएवी वियुक्तों के पूर्ण संजीन अनुकम से इनकी जानपदिक स्तर के बारे में पता चलेगा साथ ही वीपी–1 एवं वीपी–2 न्यूक्लिक अम्ल द्वारा संयुक्त रूप से प्रतिरक्षण, सीएवी से बचाव के लिए एक नया विकल्प होगा। जबकि वीपी–3 का उपयोग प्रति–अर्बुदीय औषधि के रूप में रेट्रो विषाणुजनित ट्यूमर के विरूद्ध किया जा सकता है।

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APPENDIX

A. MEDIA FOR CELL CULTURE

RPMI-1640 Medium

RPMI-1640 powder	:	10.40 g
HEPES	:	2.50 g
Sodium bicarbonate ($NaHCO_3$)	:	2.00 g
Streptomycin	:	100 mg
Penicillin	:	1 Lakh IU
Triple glass distilled water (upto)	:	1000 ml

Contents of one vial of RPMI-1640 (10.40 g) were dissolved in 500 ml of tissue culture grade distilled water. Other constituents viz. HEPES, $NaHCO_3$ and antibiotics were added in the powder form to the dissolved medium in the quantities mentioned against each and mixed completely. Final volume was adjusted to 1000 ml with distilled water. The pH was checked/adjusted to 7.4. The prepared RPMI-1640 medium was sterilized by membrane filtration (0.22 µm, Millipore), sterility tested for 48 hr and then stored at 4^0C for further use.

RPMI-1640 Growth Medium (GM)

Fetal bovine serum (FBS)	:	200 ml
RPMI-1640 medium	:	800 ml

Cell Freezing Medium

Fetal bovine serum (FBS)	:	2.00 ml
Dimethyl sulfoxide (DMSO)	:	1.00 ml
RPMI-1640 medium	:	7.00 ml

Mixed and filtered through Millipore membrane (0.22 µm).

Glasgow Minimum Essential Medium (GMEM)

GMEM	:	12.5 g
Tryptose phosphate broth (TPB)	:	2.95 g
L-Glutamine	:	300 mg
Sodium bicarbonate	:	2.75 g
Penicillin	:	2 Lakh IU
Streptomycin	:	200 mg
Distilled water up to	:	1000 ml

The medium was sterilized by Seitz filter and stored at 4^0C.

Trypsin Solution

Trypsin (Difco 1:250)	:	0.25 g
Ca^{2+} and Mg^{2+} free HBSS upto	:	100 ml

The pH was adjusted to 7.2 with addition of sodium bicarbonate and the solution was sterilized by filtration through Seitz filter and stores at 4^0C

Hanks Balanced Salt Solution

Unit I

Sodium chloride (NaCl)	:	16.0 g
Potassium chloride (KCl)	:	0.8 g
Magnesium sulphate ($MgSO_4.7H_2O$)	:	0.4 g
Calcium chloride ($CaCl_2.2H_2O$)	:	0.43 g
Distilled water	:	200 ml

Unit II

Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	:	0.297 g
Potassium dihydrogen orthophosphate (KH_2PO4):		0.120 g
Dextrose	:	4.0 g
Phenol red (0.4%)	:	10 ml
Distilled water	:	190 ml

Unit III

Sodium bicarbonate	:	1.4 g
Distilled water	:	100 ml

Units I, II and III were sterilized at 115^{0}C at 15 lbs pressure for 15 min and stored at 4^{0}C.

B. SOLUTIONS FOR LYMPHOCYTE TRANSFORMATION TEST (LTT)

Phosphate Buffered Saline (PBS) (pH 7.4)

Sodium chloride (NaCl)	:	8.00 g
Potassium chloride (KCl)	:	0.20 g
Disodium hydrogen phosphate (Na_2HPO_4 $2H_2O$)	:	1.15 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	:	0.20 g
Distilled water (upto)	:	1000 ml

Sterilized at 15 lbs pressure for 20 min and stored at 4^{0}C until used.

Trypan Blue Solution (0.4%)

Trypan blue	:	0.40 g
Phosphate buffered saline (PBS, pH 7.4)	:	100 ml

Filtered through Whatman filter paper and stored at room temperature.

MTT Dye Solution

MTT dye (SRL, India) was dissolved in PBS @ 5 mg/ml, membrane filtered and stored in dark at 4^{0}C for further use.

C. SOLUTIONS / BUFFERS FOR DNA EXTRACTION AND ELECTROPHORESIS

1M Tris-HCl (pH 7.5 & 8.0)

Tris base	:	121.40 g
Distilled water	:	800 ml

Contents mixed by stirring on a magnetic stirrer. The pH was adjusted to desired values of 7.5 and 8.0 by adding concentrated HCl. The final volume made upto 1000 ml, dispensed into aliquots, sterilized by autoclaving and stored at 4^{0}C for further use.

0.5 M EDTA (pH 8.0)

EDTA. $2H_2O$	:	18.61 g
H_2O	:	80.00 ml

Stirred vigorously on a magnetic stirrer, pH adjusted to 8.0 with concentrated NaOH (approx. 20 g of NaOH pellet), volume was adjusted to 100 ml, dispensed into aliquots and sterilized by autoclaving. Stored at 4^{0}C.

10% SDS (Sodium Dodecyl Sulphate)

SDS (electrophoresis grade)	:	10.00 g
Distilled water	:	90.00 ml

Solution was heated to 68^{0}C to assist dissolution, pH adjusted to 7.2 by adding few drops of conc. HCl. Volume was adjusted to 100 ml, and dispensed into aliquots.

5 M Sodium Chloride

Sodium chloride (NaCl)	:	29.22 g
Distilled water	:	80 ml

Solution was stirred vigorously till the salt dissolved completely, volume adjusted to 100 ml with distilled water, dispensed into aliquots and sterilized by autoclaving. Stored at 4^0C.

TNE Buffer (Tris HCl 50 mM, NaCl 150 mM, EDTA 10 mM)

Tris HCl (1M, pH 7.5)	:	5.00 ml
NaCl (5M)	:	3.00 ml
EDTA (0.5M, pH 8.0)	:	2.00 ml
Distilled Water	:	90.00 ml

Stored at 4^0C.

Lysis Buffer

Tris HCl (1 M, pH 7.5)	:	1.00 ml
EDTA (0.5M, pH 8.0)	:	2.00 ml
Triton-X 100	:	0.25 ml
Distilled Water	:	96.75 ml

Stored at 4^0C.

Proteinase K (20 mg/ml)

Proteinase K	:	20 mg
Autoclaved distilled water	:	1 ml

Aliquot and stored at -20^0C.

3 M Sodium Acetate (pH 5.2)

Sodium acetate.$3H_2O$	:	40.81 g
Distilled water	:	80.00 ml

The pH was adjusted to 5.2 with glacial acetic acid. Volume was adjusted to 100 ml prior to dispensing into aliquots and sterilized by autoclaving. Stored at 4^0C.

Tris-EDTA (TE) Buffer (pH 8.0) (Tris HCl 10 mM, EDTA 1 mM, pH 7.5)

Tris-HCl (1M, pH 8.0)	:	1.00 ml
EDTA (0.5M, pH 8.0)	:	0.20 ml
Distilled Water (upto)	:	100 ml

Autoclaved and stored at 4^0C.

Tris-Borate EDTA (TBE) Buffer (5X)

Tris-base	:	54.00 g
Boric acid	:	27.50 g
0.5 M EDTA (pH 8.0)	:	20.00 ml
Distilled water (upto)	:	1000 ml

Tris and EDTA were mixed in 800 ml of autoclaved distilled water, stirred until dissolved completely. Then boric acid was added and stirred, final volume was adjusted to 1000 ml with autoclaved distilled water. Stored at room temperature.

Ethidium Bromide (20 mg/ml)

Ethidium bromide	:	2 g
Distilled water	:	100 ml

Solution stirred on magnetic stirrer for several hours to ensure that the dye has dissolved properly. The solution was transferred to amber colored bottle and stored at 4^0C.

DNA Gel Loading Buffer (6X) (Tracking dye)

Bromophenol blue	:	0.25%
Xylene cyanol	:	0.25%
Sucrose in water (w/v)	:	40%

Stored at 4^0C.

D. SOLUTIONS FOR MOLECULAR BIOLOGICAL WORK

Luria -Bertani(LB) medium

Bact.Tryptone	:	l0g
Bact.Yeast extract	:	5g
Sodium chloride	:	l0g

pH was adjusted to 7.0 with sodium hydroxide and volume was made upto one liter with distilled water, sterilized by autoclaving.

LB Agar

LB agar was prepared by adding 1.5 % agar to LB medium, sterilized by autoclaving.

LB/Ampicillin Plates

LB agar was melted and allowed to cool to RT. Ampicillin (70pg/ml) was added.

SOB medium

Bact.Tryptone	:	2 g
Bact.Yeast extract	:	0.5 g
Sodium chloride	:	0.05 g
Potassium chloride	:	0.0186 g

pH was adjusted to 7.0 with sodium hydroxide and volume was made upto 100 ml with distilled water. Sterilized by autoclaving at 121°C at 15 psi for 15 min. Add sterile magnesium chloride to l0mM.

SOC medium

SOC medium contains 20mM Glucose in addition to SOB medium.

TSS (75ml)

2X LB	:	37.5 ml
30% PEG8000	:	25 ml
1M $MgCl_2$	:	4.5 ml
DMSO	:	3.75 ml
Sterile autoclaved distilled water	:	16.4 ml

LB and PEG were autoclaved at 121°C, 15 psi pressure for 15 min DMSO was filtered through 0.22 micrometer membrane filter.

P-I

Glucose	:	50mM
Tris HCl (pH8.0)	:	25mM
EDTA	:	l0mM

Sterilized by autoclaving at 121° C, 15psi pressure for 15 min.

P-II

Sodium hydroxide	:	200mM
SDS	:	1%

Stored at room temperature.

P- III

Potassium acetate (pH 5.5)	:	3M

pH was adjusted using glacial acetic acid and sterilized by autoclaving stored at RT.

VITAE

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Educational Qualifications:

Degree	University/College/Institute	Year	OGPA
B.V.Sc.	Madras Veterinary College, Chennai, TN.	1998	7.7/10
M.V.Sc	Indian Veterinary Research Institute Izatnagar, Bareilly, UP.	2000	8.39/10

Field of Specialization: MOLECULAR VIROLOGY

Awards/Merits :

- Senior Research Fellowship (JRF) of I.C.A.R. for Master's Study (1998-2000)
- National Eligibility Test (NET) Certificate in Veterinary Microbiology of I.C.A.R. (2001)
- Senior Research Fellowship (SRF) of I.V.R.I for Ph.D degree program. (2000-2003)

Publications:

a.) Research Papers - 12 b.) Papers Presented - 18

c.) Popular Articles - 8 d.) Awards - 2

Member of Professional/Scientific Societies:

Tamil Nadu Veterinary Council

Indian Association of Veterinary Microbiologists, Immunologists and Specialist in Infectious Diseases (IAVMI)

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