

Evidence of Avian Leukosis Virus Subgroup E and Endogenous Avian Virus in Marek's Disease Vaccines Derived from Chicken Embryo Fibroblasts

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Abstract: The aim of this study was to detect and characterize the endogenous ALVs in cell associated MD vaccine. Chicken embryo fibroblast cell associated Marek's disease vaccine was tested for possible contamination with Avian Leukosis Viruses (ALVs). Initially the vaccine cell lysate was tested for presence of group specific antigen (p27) of ALVs by ELISA and found positive for GSA. Subsequently total DNA and RNA was isolated from vaccine CEFs and analyzed by PCR and RT-PCR using primers specific for ALV subgroups A-E and J. Subgroup specific PCR and RT-PCR revealed that the CEFs were positive for ALV-E and negative for all other exogenous ALV subgroups (ALV-A, B, C, D and J). Envelope gp85 gene sequence alignment and phylogenetic analysis further confirmed that the ALV sequences found in CEFs of MD vaccine were belongs to endogenous ALV-E. Further this sequence has high homology with endogenous loci ev-1, ev-3 and ev-6. Amplification of genomic DNA with endogenous virus locus specific primers revealed that the CEFs of MD vaccine possess ev-1 and ev-6 and negative for ev-3, ev-9 and ev-21. In conclusion, the data in this study clearly demonstrated that the cell associated commercial MD vaccine tested was contaminated with an endogenous subgroup E and also possess ev-loci such as ev1 and ev-6.

Keywords: ALV-E, avian leucosis virus, chicken, env gene, ev loci, marek's disease vaccine, sequencing

INTRODUCTION

Avian Leukosis Virus (ALV) infection has been regarded as one of the important causes of economic losses in the poultry industry and therefore it is essential to establish ALV free flocks. ALVs induce a variety of tumors, reduce productivity and induce immune suppression and other production problems in affected flocks (Fadly and Nair, 2008). ALVs are classified into 10 subgroups, A-J, Based on their host range, cross-neutralization and viral interference. Chickens are the natural hosts for ALV subgroups A, B, C, D, E and J (Venugopal, 1999). On the basis of the mode of transmission ALVs are further classified as exogenous (ALV-A, B, C, D and J) and endogenous (ALV-E). Exogenous viruses can be transmitted vertically via the egg or horizontally, whereas endogenous viruses are transmitted primarily through the chicken germ line, but can also be transmitted congenitally or horizontally (Crittenden, 1976; Crittenden *et al.*, 1987; Payne, 1998). ALV-E is expressed from endogenous virus (ev) loci, which are inheritable proviral elements. At least 22 ev genes have been described in White Leghorn chickens (Crittenden, 1991). Most of these ev genes are structurally incomplete (defective) and therefore, do not encode all the sequences necessary for production of infectious

virus particles. Because ALVs can be transmitted vertically from dams to progeny embryos from infected breeder hens and tissues cultures prepared from such embryos may harbor these retroviruses and could serve as a source of ALVs contamination of poultry and other live virus vaccines produced from such ingredients (Barbosa *et al.*, 2008; Fadly *et al.*, 2006; Zavala and Cheng, 2006).

Most of the live viral poultry vaccines, including Marek's are prepared using chicken embryos or chicken embryo cell. This study was designed to characterize the endogenous ALVs in cell associated MD vaccine.

MATERIALS AND METHODS

MD vaccine: This study was conducted at Project Directorate on Poultry, Hyderabad, India during year 2011. A commercial cell associated Marek's disease virus vaccine containing serotype 2 and 3 stored in liquid nitrogen was obtained and thawed as per the instructions of the vaccine manufacturer. Three aliquots were made and one aliquot was freeze thawed three times and used for ALV group specific antigen (p27) by ELISA. The remaining two aliquots were centrifuged at 3000 rpm and cell pellets were used for DNA and RNA isolation.

Table 1: Sequences of oligonucleotide primers, targets and expected PCR product sizes

Target	Primer	Orientation	Sequence (5'-3')	Product (bp)	Reference
ALV: A-E&J	All	Forward	CGAGAGTGGCTCGCGAGATGG	2579	Silva <i>et al.</i> (2007)
	All	Reverse	ACACTACATTTCCCCCTCCCTAT		
ALV: A	H5	Forward	GGATGAGGTGACTAAGAAAG	694	Smith <i>et al.</i> (1998)
	EnvA	Reverse	AGAGAAAGAGGGGYGTCTAAGGAGA		
ALV: E	All	Forward	CGAGAGTGGCTCGCGAGATGG	1250	Silva <i>et al.</i> (2007)
	E	Reverse	GGCCCCACCCGTAGACACCACTT		
ALV: J	H5	Forward	GGATGAGGTGACTAAGAAAG	544	Smith <i>et al.</i> (1998)
	H7b	Reverse	CGAACCAAAGGTAACACACC		

Detection of ALV group specific antigen (p27): The cell lysate was centrifuged at 3000 rpm for 5 min and the supernatant was tested for group specific antigen (p27) using antigen ELISA kit (IDEXX Laboratories Ptv. Ltd., USA) (IDEXX). Briefly, 100 µL of sample was added into an anti-p27 antibody-coated well in duplicate and incubated at room temperature for 60 min. After washing the wells 4 times with wash solution, 100 µL of Rabbit anti p27 IgG-HRP, conjugate added to the wells and again incubated at room temperature for 60 min. Unreacted conjugate was removed by washing 4 times with distilled water and a substrate containing the chromogen 3, 3', 5, 5'-Tetramethylbenzidine (TMB) was added to the wells and incubated for a further 15 min at room temperature. The relative intensity of the color which developed after 15 min was known to be directly proportional to the quantity of p27 antigen in the sample. Absorbance (OD) for each well was measured at 650 nm using an ELISA reader. The Sample to Positive (SP) ratio was calculated using the formula $SP\ ratio = [(sample\ OD) - (negative\ control\ OD)] / [(mean\ positive\ control\ OD) - (mean\ negative\ control\ OD)]$. The positive and negative control sera provided in the kit were run in duplicate. Sample showing SP ratios of ≥ 0.20 were considered positive for ALV gs-antigen.

Total DNA isolation and PCR analysis: DNA was isolated by standard phenol-chloroform method. Briefly lysis buffer (50 mM Tris, 20 mM EDTA, 2% SDS and 200 µg proteinase K/mL) was added to the cell pellet and incubated at 37°C for 3 h. DNA from lysed cells was extracted using an equal volume of phenol: chloroform: isoamylalcohol (25:24:1 v/v) (SRL) by vortex mixing and centrifugation at 14000 rpm for 5 min. The DNA was precipitated in 1 volume of 100% isopropyl alcohol at room temperature for 15 min. The precipitation mixture was centrifuged at 14000 rpm for 15 min at room temperature. The DNA pellet was washed in 70% isopropyl alcohol, dried at 37°C, resuspended in nuclease-free water and stored at 4°C and used for PCR analysis. The primers used for direct DNA PCR of ALVs are indicated in Table 1.

Each 25 µL reaction mixture consisted of 2.5 µL 10x Taq DNA polymerase buffer (Sigma), 1.5 µL 25 mM MgCl₂, 0.5 µL 10 mM each dNTP (Fermentas), 0.5 µL 20 pm of each primer, 1U of Taq DNA

polymerase (Sigma) and 1 µL of template DNA. Reaction volumes were made up to 25 µL using nuclease free water.

RNA isolation and RT-PCR analysis: For extraction of RNA from cell pellet was mixed with 1 mL Trizol reagent in a micro-centrifuge tube and incubated at room temperature for 15 min. After incubation 200 µL of chloroform was added, mixed thoroughly, incubated at room temperature for 10 min and centrifuged at 13000 rpm for 15 min at 4°C. RNA in the aqueous phase was precipitated with an equal volume of cold isopropanol and incubated at -20°C for 20 min. Then pelleted by centrifugation at 13000 rpm for 10 min at 4°C and washed once with 1 mL of cold ethanol. After drying, the RNA pellet was resuspended in 30 µL of DEPC water (Fermentas) and soon prepared complementary DNA.

Complementary DNA (cDNA) was synthesized using standard protocol. Briefly, 5 µL of total RNA was mixed with 1 µL oligo (DT) primer (100 µM) and 6 µL of RNase free water and incubated at 70°C for 5 min. The mixture was snap cooled on ice and then 4 µL 5X RT buffer, 2 µL dNTP mix (10 mM each), 1 µL ribolock (40 U/µL) was added and incubated at 37°C for 5 min. In the final step 1 µL (200 U/µL) reverse transcriptase (Fermentas) was added to the tube mixed and incubated at 42°C for 60 min, 70°C for 10 min and stored at -20°C until use. All the incubation steps were carried out in thermo cycler. The sequences of the oligonucleotide primers used in the study are shown in Table 1 (Silva *et al.*, 2007; Smith *et al.*, 1998). All the primers were synthesized by Integrated DNA technologies, USA.

Detection of ev loci in MDV CEFs: Endogenous *ev* loci present within the genomic DNA of the chicken embryo fibroblasts products in original vaccine vials were identified based on methods previously described by Benkel (1998). Briefly, genomic DNA was extracted as described above. The PCR primers used to detect *ev* loci were directed against the *ev*LTRs and the unique flanking sequences of integration sites. Amplification of the targeted sites produced fragments of expected lengths indicative of the presence or absence of specific *ev* locus in chicken cells. Five different *ev* loci, including *ev*1, *ev*-3, *ev*-6, *ev*-9 and *ev*-21 were tested.

The presence or absence of the *ev* locus in CEF cells was determined based on the specific size of the amplification fragments (Benkel, 1998).

Agarose gel electrophoresis: PCR products were separated on 1.5% (w/v) agarose (Himedia) gels containing 0.5 µg/mL ethidium bromide and visualized using gel documentation system (Bio-Rad).

Nucleotide sequencing: The gp85 of env gene of ALV-E was amplified using forward primer ALV Egp85- F (5'-TGGTGTCTTGGTCTTGTGT GAGG-3') and reverse primer ALVEgp85-R (5'-GCGATGAATTGGCAA GCACCTTG -3'). The PCR conditions used were an initial template melting at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min. A final elongation step was at 72°C for 10 min. The DNA sequencing was performed by Chromous biotech using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer in accordance with the specifications of manufacturer.

Analysis of nucleotide and deduced amino acid sequences: The gp85 sequence was generated from forward and reverse sequence data obtained from Chromous biotech Ltd. The BLAST algorithm at the National Centre for Biotechnology Information (NCBI) was used for homology searches against GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>). Nucleotide and protein multiple sequence alignments were performed using the ClustalW in Lasergene DNASTAR software. Phylogenetic relationships estimated using program in the Lasergene DNASTar software. The gene bank accession numbers of sequences used in analysis include:

- M37980 (ALV-A)
- AF052428 (ALV-B)
- J02342 (ALV-C)
- D10652 (ALV-D)
- EF467236 (ALV-E)
- Z46390 (ALV-J)
- AY013303 (ev1)
- AY013304 (ev3)
- AY013305 (ev6)

RESULTS

Detection of ALV group specific antigen (p27) in CEFs: CEF cell lysate was screened for presence of ALV group specific antigen by ELISA. The CEF lysate was found positive for ALV group specific antigen (p27) indicating presence of ALV or endogenous ALV sequences expressing p27 in the vaccine derived from CEFs.

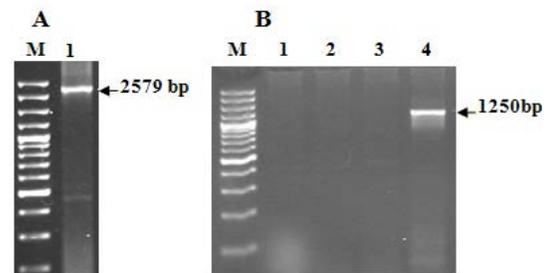


Fig. 1: A. PCR with universal primers (AllF/AllR) which amplify ALV subgroups A, B, C, D, E and J. B. PCR with subgroup specific primers
M: DNA Marker; lane 1: ALV-A specific primers; lane 2: ALV-B and D specific Primers; lane 3: ALV-D specific primers; lane 4: ALV-E specific primers

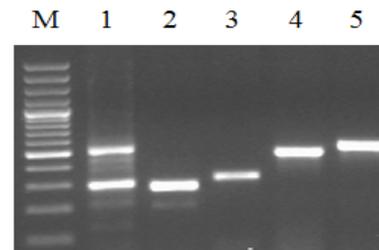


Fig. 2: Typing of *ev* loci in CEF DNA by locus-specific PCR analysis

Test results for each locus show diagnostic PCR fragments of specific sizes that indicate the presence or absence of the locus; Lane M: DNA molecular size marker; lane 1: *ev-1* test result showing fragments for the presence (295 bp) and absence (505 bp) of the locus, indicating heterozygosity (+/-); lane 2: *ev-3* test result showing a fragment (270 bp) for the absence of the locus (-/-); lane 3: *ev-6* test result showing a fragment (300 bp) for the presence of the locus (+); lane 4: *ev-9* test result showing a fragment (450 bp) for the absence of the locus (-/-); lane 5: *ev-21* test result showing a fragment (510 bp) for the absence of the locus (-/-)

Detection of ALV RNA and proviral DNA in CEFs of MD vaccine:

The DNA and RNA isolated from vaccine CEFs were tested for ALV specific sequences by PCR and RT-PCR, respectively, using universal primer set ALV-ALLF and ALV-ALLR primers which detect ALV-A to E and J subgroups. Both PCR and RT-PCR analysis revealed presence of ALV specific sequences in the CEFs of MD vaccine (Fig. 1). The subgroup identification was carried out by PCR and RT PCR using subgroup specific primers: H5/EnvA, ALLF/BDR, ALLF/CR, ALLF/ER and H5/H7b which amplify ALV subgroup -A, -B, -C, -D, -E and -J, respectively. PCR and RT-PCR analysis by subgroup specific primers revealed that the DNA and RNA was

Table 2: Nucleotide and amino acid identities of gp85 of ALV from MD vaccine with ALV subgroups and selected *ev* loci

Nucleotide identity (%)										
	MDVac	ALV-A	ALV-B	ALV-C	ALV-D	ALV-E	ALV-J	ev1	ev3	ev6
MDVac		87.1	85.8	88.1	87.4	99.0	48.7	99.3	99.0	98.9
ALV A	83.3		82.2	85.2	84.3	87.3	48.1	87.1	87.2	87.2
ALV B	80.0	77.7		83.9	86.8	85.6	48.2	85.9	86.0	86.0
ALV C	84.1	83.0	78.9		85.6	87.8	47.4	88.4	88.3	88.3
ALV D	84.7	82.9	87.8	83.4		87.2	47.4	87.6	87.7	87.3
ALV E	98.5	83.0	79.4	83.4	84.1		48.9	99.3	99.0	98.9
ALV J	39.3	38.1	38.6	39.9	38.2	38.6		48.9	49.1	48.6
ev1	99.1	83.0	79.7	84.4	84.4	98.5	39.3		99.7	99.2
ev3	98.5	83.3	80.3	84.4	85.0	97.8	38.9	99.4		99.1
ev6	97.8	83.0	79.7	83.5	84.1	97.5	38.9	97.8	97.8	

Amino acid identity (%)										
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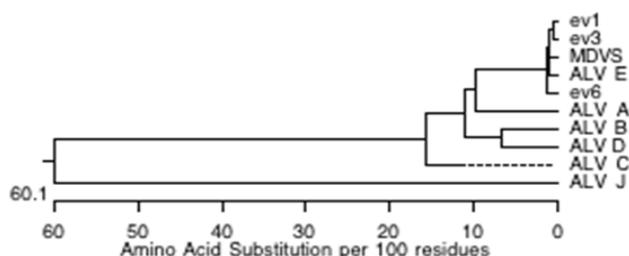


Fig. 3: Phylogenetic relationship of ALV-E from MD vaccine and representative exogenous ALV subgroups and endogenous virus loci based on the gp85 amino acid sequence

positive for endogenous ALV-E and negative for other subgroups ALV-A, B, C, D and J.

Detection of *ev* loci in CEFs of cell associated MD vaccine: The results of locus-specific PCR analysis of CEF DNA identified the presence of *ev-1* and *ev-6* ALV-E loci. No evidence of *ev-3*, *ev-9* and *ev-21* was found (Fig. 2). The CEF DNA was found to be heterozygous for *ev-1*, as indicated by the presence of both positive (295-bp) and negative (505-bp) PCR fragments (Fig. 2). The fragment size 300 bp in lane 3 indicate insertion of *ev-6*, whereas the fragment size 270 bp in lane 2, 450 bp in lane 4 and 510 bp in lane 5 represent absence of *ev-3*, *ev-9* and *ev-21*, respectively.

Sequence analysis: The nucleotide sequence of gp85 was determined and compared with NCBI gene bank sequences of reference strains belonging to subgroups A, B, C, D, E and J and *ev-1*, *ev-3* and *ev-6* endogenous loci. The gp85 nucleotide sequence covers 865 nucleotides which include variable and hyper variable regions of gp85 region (NCBI Gene Bank Accession No: JN977128). The percent identity of nucleotide and translated amino acid sequences are presented in Table 2. The sequence identity of gp85 of ALV from MD vaccine was ranged between 85.8 to 88.1% with ALV subgroups A to D and only 48.7% with ALV-J. However, the identity percent with ALV-E and endogenous loci *ev-1*, *ev-3* and *ev-6* was 99.0, 99.3, and 98.9%, respectively, indicating that the present ALV sequence is more identical with ALV-E and endogenous ALV loci.

99.0 and 98.9%, respectively, indicating that the present ALV sequence is more identical with ALV-E and endogenous ALV loci.

To assess the evolutionary relationship of ALV-E sequence obtained from MD vaccine, a phylogenetic tree of ALV subgroup sequences representing the gp85 coding region was constructed. Phylogenetic analysis revealed that the ALV E detected in this study clustered in a single group with ALV-E and *ev-1*, *ev-3* and *ev-6*, which, further indicates that the ALV obtained from CEFs of MDV is closely related to and belongs to ALV subgroup-E. The other five subgroups of ALV (A, B, C, D and J) compared were in 5 separate groups (Fig. 3).

DISCUSSION

Marek's Disease (MD) is a lymphomatous and neuropathic disease of chicken caused by an Alpharetrovirus (Payne and Venugopal, 2000). MD is mainly prevented by vaccinating chickens *in ovo* or at 1 day of age using monovalent cell free (serotype 3) or bivalent cell associated (serotype 2 and 3) vaccines. These vaccines are produced in specific pathogen free chicken embryo fibroblast cell cultures. Avian leukosis viruses have been associated with wide range of neoplastic and non neoplastic conditions in poultry causing significant economic losses to the poultry industry. No specific treatments or vaccines are available against ALV infection. The current approach

is the eradication of exogenous ALV from elite egg-type and meat-type breeding stock by primary breeding companies, to produce infection free commercial grandparent or parent breeding progeny (Fadly and Nair, 2008).

Because ALVs can be transmitted vertically from dams to progeny embryos from infected breeder hens and tissues cultures prepared from such embryos may harbor these retroviruses and could serve as a potential source of ALVs contamination of poultry and other live virus vaccines produced from such ingredients (Fadly *et al.*, 2006; Barbosa *et al.*, 2008; Zavala and Cheng, 2006). If present as contaminants in poultry vaccines, these viruses present a disease hazard to poultry. The aim of this study was to test cell associated Marek's disease vaccine for contamination of ALV and to determine the variation in at nucleotide and amino acid levels. There are numerous cases of documented contaminated vaccines derived from chicken embryos and chicken embryo cell cultures intended for human (Hussain *et al.*, 2003; Johnson and Heneine, 2001; Tsang *et al.*, 1999), poultry (Mohamed *et al.*, 2010; Silva *et al.*, 2007; Fadly *et al.*, 2006; Zavala and Cheng, 2006) and other. Because ALVs are transmitted from dams to offspring, embryos and tissue cultures prepared from such embryos may harbor endogenous and exogenous ALVs and may serve as a source of ALV contamination of poultry and other vaccines.

Natural and experimental mixed infections with avian retroviruses and MDV have been described previously (Bacon *et al.*, 1989; Davidson *et al.*, 2002; Fynan *et al.*, 1993). Coinfection with such two viruses in cells may promote replication and pathogenesis of one or both virus types (Pulaski *et al.*, 1992). Serotype 2 MDV strains may enhance expression of bursal lymphomas in dually infected chickens partially as a result of enhanced avian leucosis viral gene expression (Bacon *et al.*, 1989; Marsh *et al.*, 1995; Pulaski *et al.*, 1992). Hence, inoculation of any vaccine contaminated with ALV and containing serotype 2 MDV could potentially result in a higher incidence of ALV related lymphomas.

The presence of ALV subgroup E and endogenous loci, *ev-1* and *ev-6* were detected in CEFs of cell associated MD vaccine. The PCR products of ALVE were further confirmed by sequencing of the gp85 region of the contaminant ALV, which suggested 99% homology with published sequence of subgroup E ALV.

PCR and RT-PCR amplification of proviral DNA and viral RNA indicated that the contaminant was a subgroup E virus. Subsequent sequencing confirmed that the contaminant was an ALV-E. Aligning the gp 85 sequences from the contaminating virus with sequences

of exogenous (ALV-A to D and J) and endogenous ALV-E and *ev-1*, *ev-3* and *ev-6*) taken from Gen Bank clearly showed that the gp85 was not typical of that found in exogenous ALVs, such as ALV-A, B, C, D and J, but rather was closely related to gp85 found in subgroup E virus and endogenous viral loci *ev-1*, *ev-3* and *ev-6*. These results coincide with reports of the detection of ALV and EAV particles in live vaccines derived from chicken embryo fibroblasts has been reported previously in USA (Fadly *et al.*, 2006; Barbosa *et al.*, 2008; Zavala and Cheng, 2006), Egypt (Mohamed *et al.*, 2010) and Cuba (Acevedo *et al.*, 2009). Although ALV E is not known to cause tumours in chickens, its presence in vaccines is not favorable, because it has negative effect on the immune response of chickens to infection with exogenous ALV and can cause confusion in diagnosing exogenous ALV infection (Crittenden *et al.*, 1987).

Generally poultry breeding companies do not expect to contain any ALV in the cells that are injected into their chicken lines. Although endogenous ALVs are non-oncogenic they may contribute emergence of variants or new subgroup viruses with new disease producing properties, through recombination with exogenous ALVs (Bai *et al.*, 1995). Homologous and non-homologous recombination between retroviral sequences and also between retroviral sequences and cellular RNA has been described (Hajjar and Linial, 1993; Girod *et al.*, 1996). The potential of ALV for undergoing recombinations is no surprise, as emergence of the original ALV-J isolate has been attributed to recombination occurring between an exogenous virus and an endogenous retroviral sequence (Bai *et al.*, 1995). Eradication of exogenous ALVs has become a priority in recent years. This is necessary to reduce the potential for exogenous viruses to undergo recombination with other exogenous virus, or with endogenous retroviral sequences. Interestingly, elimination of one potential source of recombination, the endogenous ALV-E sequences, has recently become a more realistic possibility (Bacon *et al.*, 2004). Endogenous viral genes are not required for survival and normal development in the chicken but their presence in the genome can have direct or indirect effects on immune responsiveness and performance (Crittenden, 1991; Gavora *et al.*, 1991).

Current regulations require that vaccines be produced from ingredients lacking exogenous ALV only, namely ALV-A, ALV-B and ALV-J. Because endogenous ALV has been shown to increase susceptibility of chickens to infection and tumors induced by exogenous ALV, the presence of endogenous ALV in poultry vaccines may not be desirable. In addition ALV infection cause

immunosuppression in the host that allows the chance of secondary infections by opportunistic bacterial agents and may also results in poor response to vaccines. Embryonic infection with ALV-E causes a more persistent viremia and more neoplasms following infection with exogenous ALV due to depression of humoral immunity (Crittenden *et al.*, 1987). Further, all ALVs including endogenous ALV-E encode a major group specific antigen (p27) which has immunosuppressive effects on host defense (Pang *et al.*, 2010).

In conclusion, these findings reveal that the cell associated commercial MD vaccine tested was contaminated with an endogenous ALV subgroup E and also possess ev-1 and ev-6 insertions in the genome of CEFs. More extensive studies are needed to assess the risk of infection with contaminating ALVs in chickens.

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