BJVP
Brazilian Journal of Veterinary Pathology

Original full paper

Detection of reticuloendotheliosis virus by immunohistochemistry and in situ hybridization in experimentally infected chicken embryo fibroblasts

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Submitted January 14th 2009, Accepted February 10th 2009

Abstract

Reticuloendotheliosis virus (REV) infection can result in immunosuppression, runting syndrome, high mortality, acute reticular cell neoplasia, or T- and/or B-cell lymphoma, in a variety of domestic and wild birds. Histopathological changes of reticuloendotheliosis (RE) are not sufficient to differentiate it from Avian Leukosis (AL) and Marek's disease (MD). Currently there are no available diagnostic methods for detection of active REV infection. In order to develop immunohistochemistry (IHC) and *in situ* hybridization (ISH) assays for detection of REV active infections, experimentally inoculated formalin-fixed and paraffin embedded DF-1 chicken embryo fibroblasts were used as an infection model. IHC and ISH assays proved to be efficient for the detection of several REV strains, and to differentiate those strains from representative strains of the avian leukosis/sarcoma group of retroviruses (ALSV).

Key Words: Immunohistochemistry, *in situ* hybridization, Reticuloendotheliosis, chicken, fibroblast.

Introduction

Reticuloendotheliosis virus (REV), currently classified as a *Gammaretrovirus* (3), consists of a group of pathogenic retroviruses which shares structural, morphological and antigenic similarities to the mammalian Type C retroviruses (2). The REV's are immunologically, morphologically, and structurally unrelated to the viruses of the avian leukosis/sarcoma group (ALSV) of retroviruses (22, 25, 33). REV's can be classified by the replication capacity either as defective (acutely oncogenic) or nondefective (associated helper) viruses (33). Representative strains of the REV group include the defective REV-T and the nondefective REV-A, both originally isolated from turkeys (6). Additionally, other REV strains have been isolated from other avian species including geese, pheasants, peafowl, Japanese quail, greater prairie chickens (*Tympanuchus cupido pinnatus*), and Attwater's prairie chickens (*Tympanuchus cupido attwateri*) (3, 4, 37). Natural or experimentally induced reticuloendotheliosis (RE) has also been reported in a variety of wild birds (15, 20, 23, 33).

Clinical outcome of infection can range from runting syndrome to acute reticular cell neoplasia or T-cell and/or B-cell lymphomas that resemble Marek's disease (MD) and Avian Leukosis (AL) (33, 35). REV results in an oncogenic and immunosuppressive disease, increasing condemnation due to neoplasia and secondary infections,

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and contaminating chicken cell-derived materials used as substrate for laboratory techniques, such as propagation of vaccines (8, 31, 33). The virus is widespread and clinical disease can occur by vertical, but non-genetic (2), or horizontal transmissions, due to direct or indirect contact with infected birds. Humans (8) and insects (10, 21, 34) can mechanically transport the virus. However, most cases are related to vaccination of young birds. REV can integrate into the genome of large DNA viruses, including fowl pox and MD viruses, which are commonly controlled by vaccination in commercial poultry flocks worldwide. Those vaccine strains carrying a nearly intact REV provirus are most likely to cause disease in the field (1, 12, 13, 16, 18, 19, 27, 28, 30, 32, 33, 36).

Considering that REV can induce either T or B-cell lymphomas, histopathology and lymphocyte markers do not allow a differential diagnosis with MD or AL, which cause T-cell and B-cell transformation, respectively (8). Outbreaks of B-cell lymphomas induced by natural REV infections in commercial chicken flocks have often been reported, whereas the T-cell lymphomas are more usually associated with experimental inoculations (11). There are no diagnostic methods currently available for detection of active REV infections (33).

The aim of this study was to develop immunohistochemical and *in situ* hybridization assays for detection of REV (17, 24, 26).

Material and methods

Cells line, viruses and inoculation

The CEF UMNSAH/DF-1 cell line, a spontaneously immortalized chicken fibroblastoid cell line derived from 10 day-old East Lansing Line zero (ELL-0) embryos (ATCC:number CRL-12203; American Type Culture Collection, Manassas, VA, USA), was used in this study. Cells were grown in 100mm cell culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. Confluent monolayers were inoculated with 5 X 10^3 TCIU (tissue culture infectious units) of REV. Five REV strains were used in this study, as well as representative strains of AL virus (Table 1). Uninfected cells inoculated with sterile neutral phosphate buffered saline (PBS) were used as negative controls for possible nonspecific staining.

Four days after inoculation, the cells were resuspended by trypsinization, centrifuged at 1,000 g for 5 minutes, and the pellets were embedded in 1% agarose gel, which were then fixed in 10% formalin for 24 hours, processed for paraffin embedding, sectioned at 4μm, and mounted onto positively charged slides.

Immunohistochemistry

Unstained sections were heated at 60°C for 15 minutes and deparaffinized in Hemo-De (PMP Medical Industries, Irving, TX, USA) for 20 minutes. The slides were dried at 37°C for 20 minutes, and the cell preparations encircled by hydrophobic pen. Antigen retrieval was accomplished through microwaving in citrate buffer for 10 minutes at full power. The sections were blocked with casein solution. A commercially available polyclonal inactivated avian reticuloendotheliosis antiserum (Charles River Laboratories, Wilmington, MA, USA) was used as primary antibody in a concentration of 1:5000 in PBS, and incubated at 4°C overnight. A secondary biotinylated anti-chicken antiserum (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:5000 in PBS was then applied, and the slides were incubated in a humid chamber at 37°C for 1 hour. For detection, the Vectastain avidin-biotin complex-alkaline phosphatase (ABC-AP) kit (Vector Laboratories) was used, followed by a commercially available ABC-AP substrate kit (Vector Red; Roche Diagnostics, Indianapolis, IN, USA), as per the manufacturer's instructions. Slides were then lightly counterstained with hematoxylin for 12 seconds and coverslipped for a permanent record.

Table 1 - Reticuloendotheliosis and Avian Leukosis virus strains used in this study.

Sample	GenBank accession	Vírus Description
Rev-A	NC_0069348295	Reticuloendotheliosis virus
Rev-T	K00555 X02759	Reticuloendotheliosis virus, proviral, oncogene - v-rel
Rev APC 566	DQ 387450	Reticuloendotheliosis virus
Rev RU	NA*	Reticuloendotheliosis virus
Rev 397 A	NA*	Reticuloendotheliosis virus
ALV-J PDRC	Z46390	Sub-group J (HPRS-103 ALV)
ALV-J ADOL	AY 027920	Sub-group J (ADOL 7501)
RAV-1 Sub-group A	NC_001408	Rous sarcoma virus
RAV-2 Sub-group B	M14902	Rous sarcoma virus
RAV-49 Sub-group C	AF033808	Rous sarcoma virus
RAV-50 Sub-group D	M22730	Rous sarcoma virus (strain Schmidt-Ruppin)
RSV-B	NA*	Rous sarcoma virus

In Situ hybridization

The protocol used in this study was modified from a previously described ISH method (5). Total RNA from the REV strain APC-566 was used for synthesis of the riboprobe. This REV strain contains two identical long terminal repeats (LTR) and a complete set of genes including gag, gag-pol and env and high sequence similarity with gag and pol genes from other REV strains (3, 4, 37). First strand cDNA was synthesized using 2μg of RNA, an oligo-dT(18) primer, 0.25mM dNTPs, and Superscript reverse transcriptase (Invitrogen, Carlsbad,

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CA, USA), according to the manufacturer's instructions. Three pairs of primers were designed based on the REV genome sequence (GenBank accession number NC_006934) (Table 2). PCR amplification of full-length open reading frames was performed using the following parameters: 94°C for 5 minutes; 30 cycles of 94°C 30 seconds, 55°C for 30 seconds, and 72°C 30 seconds, followed by a final extension step at 72°C for 7 minutes. PCR products were purified using a commercial PCR purification Kit (Qiagen, Valencia, CA, USA), ligated into the pGEM-Teasy vector (Qiagen), and the resulting plasmids were transformed into *Escherichia coli* by heat shock. Positive colonies were identified by PCR, and then further confirmed by sequencing of both strands of the insert. Plasmid DNA was purified with a commercial mini-prep kit (Promega, Madison, WI, USA), and cleaved with SacII. Linearized constructs were used for synthesis of the riboprobes by in vitro transcription with SP6 RNA polymerase. Three anti-sense riboprobes were generated, namely REVgag-500, REVgag-259, and REVgag-151 (Table 2). Sense riboprobes were also synthesized and used as negative controls.

Table 2 - Primers and PCR product sizes used for generation of the riboprobes.

Probe	Sequence (5'-3')	Size*
VRE-gag Sense 1	TGCGCAAGTTATGTGAGTCGGA	448**
VRE-gag Sense 2	AGGCGGCCCGTGCTCCCCTCAGC	259
VRE-gag Anti-Sense 2	CCCGTTCCCCAGTTTCCCTAAGGGG	
VRE-gag Sense 3	GGAGGGCTCAATTCCTGGATG	151
VRE-gag Anti-Sense 3	CCACTTCGGGTGTGGGGAGGGCTC	

* PCR product size (bp).
** The product indicated for the primer "VRE-gag Sense 1" (448 bp) was obtained amplifying with the primer "VRE-gag Anti-Sense 2".

Unstained slides were incubated at 60°C for 20 minutes, and deparaffinized in Hemo-De (PMP Medical Industries) for 20 minutes. The slides were allowed to dry at 37°C for 20 minutes and the cells were encircled with a hydrophobic pen. The cells were then re-hydrated in PBS plus 5mM $MgCl_2$ for 10 minutes at room temperature. The slides were rinsed in 0.2M Tris pH7.5 plus 0.1M glycine for 10 minutes at room temperature, followed by 15 minutes of incubation with proteinase K in 10mM Tris pH7.5 with 2mM $CaCl_2$ at 37°C. The cell preparations were washed with 0.2M Tris pH7.5 with 0.1M glycine for 2 minutes, followed by incubation with the pre-hybridization solution (5X standard sodium citrate, 50% formamide, 5% blocking reagent (Roche Diagnostics, Indianapolis, IN, USA), 1% N-lauroylsarcosine, 2% sodium dodecyl sulfate) for 1 hour at 42°C. Each slide was incubated with 2µl of the riboprobe diluted in 70µl of pre-hybridization solution. The slides were then covered with siliconized cover slips, the edges of which were sealed with nail hardener, and incubated overnight at 42°C. Cover slips were removed and sequential stringent washes were performed with 2XSCC plus 1% SDS for 30 minutes at 50°C; 1XSCC plus 0.1% SDS for 30 minutes at 50°C; 1XSSC for 30 minutes at room temperature, 0.1XSSC for15 minutes at room temperature; and buffer 1 (100mM Tris–HCl pH7.6, 150mM NaCl) for 5 minutes at room temperature. The slides were then incubated with a 1:300 dilution of anti-digoxigenin-AP (Roche) with 2% sheep serum in buffer 1 for 2 hours at 37°C. Sequential washes were performed with 3 washes with buffer 1 for 15 minutes at room temperature, and buffer 3 (100mM Tris–HCl pH9.5, 100mM NaCl, 50mM $MgCl_2$) for 5 minutes at room temperature. The substrate/chromogen (nitroblue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP, Roche) was added. One drop of (5 mM) levamisole (Vector) was added to quench endogenous alkaline phosphatase. Development progressed for 45 minutes to 1 hour and slides were counterstained lightly with hematoxylin and coverslipped for a permanent record.

Results

With both IHC and ISH, strong positive labeling was observed, both in the nucleus and cytoplasm of cells inoculated with all REV strains (Fig. 1). All three REV anti-sense riboprobes yielded positive staining in the nucleus and cytoplasm of REV-inoculated cells and not in any ALV-J-inoculated cells. The riboprobe REVgag-500 resulted in the strongest signal. All PBS-inoculated and ALSV-inoculated cells were consistently negative without any significant background staining (Fig. 2). Also, sense probes did not result in any positive staining.

Discussion

The results of this study demonstrate the possibilities of IHC and ISH assays for detecting active REV infections. IHC and ISH yielded very similar results, with both assays being able to detect cells infected with different REV strains. Both techniques indicated the presence of antigens or viral RNA both in the nucleus and cytoplasm of infected cells, which is compatible with the biology of retroviruses, in which replication stages take place both in the nucleus and cytoplasm (7). One of the riboprobes, namely REVgag-500, resulted in the strongest signal, which may be due to the size of this probe (448 bp) that is larger than the other riboprobes used in this study. These results support the notion that DF-1 CEF cell line is susceptible to REV infection and replication, and it is a suitable model for REV infection.

However, the results obtained in this study cannot be directly extrapolated to naturally infected birds since in

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REV-induced lymphomatous diseases the viral genome is present in neoplastic cells as proviral DNA integrated into

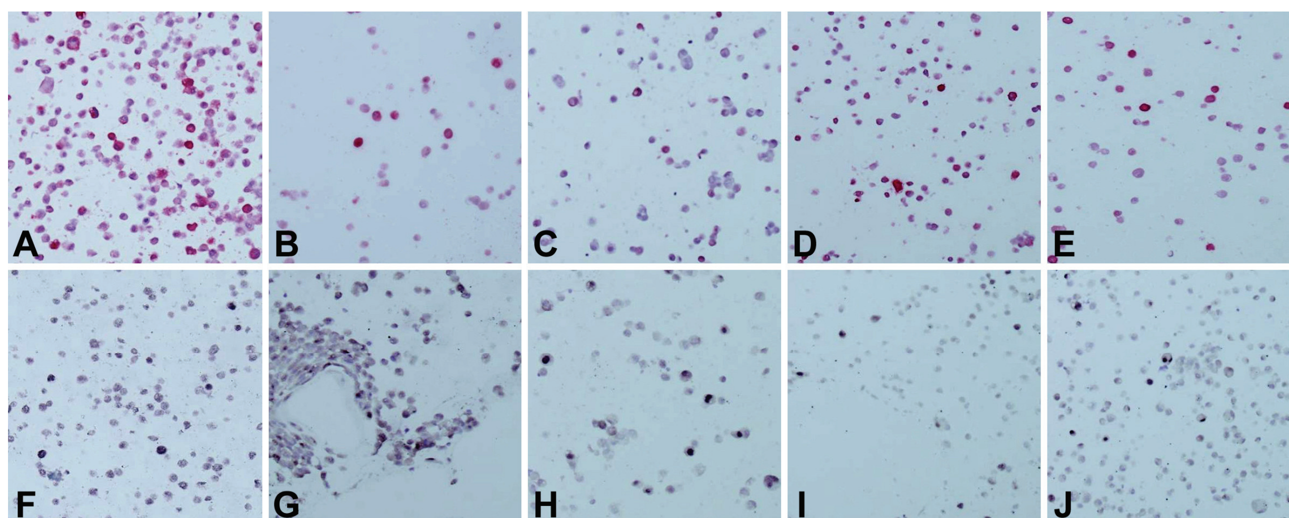

Figure 1 - Cultured formalin fixed and paraffin embedded DF-1 chicken embryo fibroblasts. A-E: cells inoculated with the REV strains A, T, RU, APC 566, and 397 A, respectively, positively labeled by immunohistochemistry; F-J: cells inoculated with REV strains A, T, RU, APC 566, and 397 A, respectively, positively labeled by in situ hybridization, using riboprobe REV gag-500.

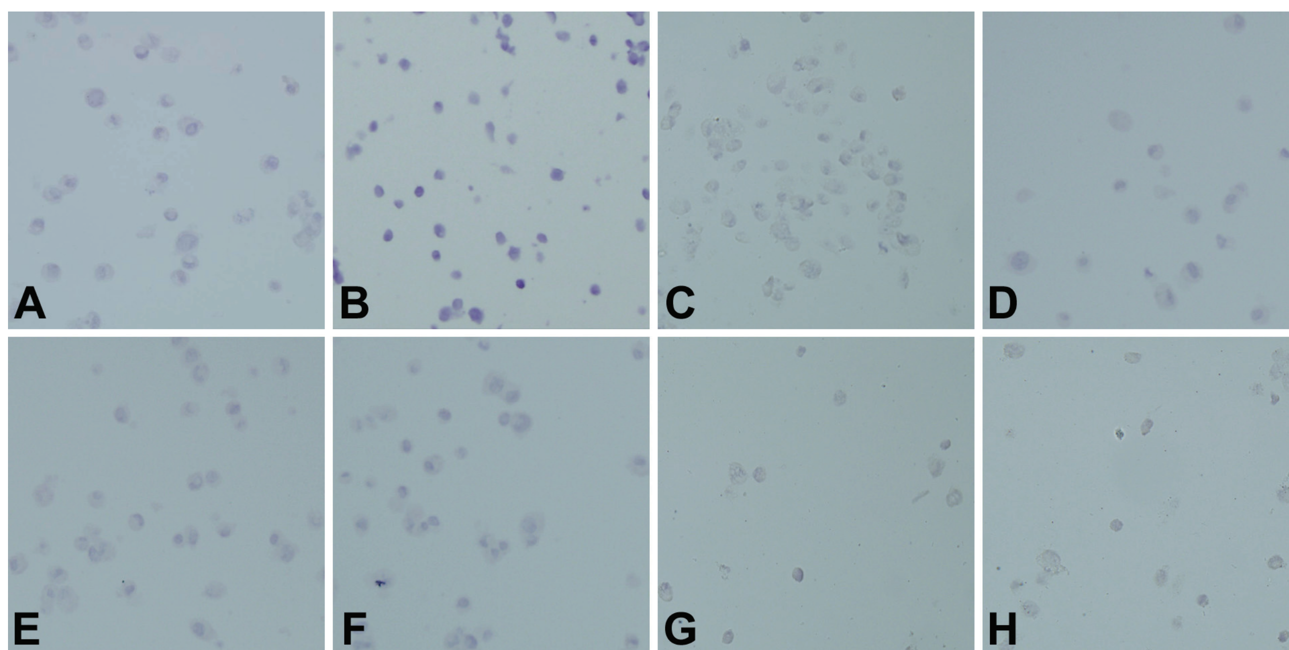

Figure 2 - Cultured formalin fixed and paraffin embedded DF-1 chicken embryo fibroblasts processed for immunohistochemical labeling of REV. A: negative control cells inoculated with sterile PBS; B-H: cells inoculated with ALV-J PDRC, ALV-J ADOL, RAV-1, RAV-2, RAV-49, RAV-50, and RSV-B strains, respectively.

the transformed host cell genome. Therefore, during the stage of clinical lymphomatous disease, transformed cells are not associated with active viral replication, and they are generally free of viral particles (29). PCR, virus isolation, and indirect immunofluorescence are techniques that have been used to demonstrate REV infection (8, 9, 33). However, viral activity, tropism, and pathogenicity can only be evaluated by using techniques that allow for *in situ* localization of the virus, such as ISH and IHC. In addition, IHC and ISH are suitable for formalin fixed and paraffin embedded tissue samples, allowing retrospective studies in archived tissues.

Acknowledgments

VLSLS is a recipient of a scholarship from CAPES (Brasilia, Brazil) under the CAPES/FIPSE exchange program. RLS is a recipient of a research fellowship from CNPq (Brasilia, Brazil).

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