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# MULTIPLE INFECTION OF CHICKENS AND TURKEYS WITH AVIAN ONCOGENIC VIRUSES: PREVALENCE AND MOLECULAR ANALYSIS

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**Summary.** – The avian herpesvirus, Marek's disease virus (MDV) and several retroviruses, reticuloendotheliosis virus (REV), avian leukosis virus (ALV) (chickens) and lymphoproliferative disease virus (turkeys) are oncogenic and immunosuppressive agents. These viruses were detected either alone, or in various combinations in blood and tumor DNAs of commercial birds using PCR. We present a 5-year retrospective study that included 207 chicken and 52 turkey flocks. Of these, 32 chicken and 18 turkey flocks were negative. Of the positive chicken and turkey flocks 76% and 75%, respectively, had a single, while the rest, 24% and 25%, had a multiple virus infection. In the chickens of the multiple virus-infected flocks, 14% and 17% of the blood and tumor DNAs carry dual MDV and REV and/or ALV sequences, that is about 30% of the PCR-positive, and about 5% of the total DNAs analysed. Multiple virus sequences were detected only in the turkey blood DNAs – 11% of 84 samples. Following that quantitation we aimed to analyse the molecular status of the retrovirus sequences in order to determine whether retrovirus sequences were integrated into the herpesvirus genome. We focused on the MDV *Bam*H1-H 132 bp tandem repeat fragment proximity using a combined PCR (cPCR) to identify chimeric PCR products. That included amplification with heterologous combinations of the MDV and retroviral LTR primers. In 13 of 35 DNAs that had both MDV and retrovirus sequences new products were produced. Of 4 MDV+REV chimeric products that were sequenced, one was homologous to the Chicken Repeat element 1 non-LTR type retrotransposon. No evidence for a retrovirus LTR integration was found in the 132 bp repeat proximity, but in two of these products we detected nucleotide stretches of 20 bp and 21 bp with a 70% and 71% homology to the REV-LTR. Also, the amplification of the chimeric products using a retrovirus primer denoted that at least short nucleotide stretches homologous to retroviral LTR primer were present in these DNAs, and that they might resemble ancient retroviral insertions, as previously demonstrated (Isfort *et al.*, 1992).

**Key words:** avian oncogenic viruses; PCR; herpesviruses; retroviruses; molecular interactions; multiple virus infection

## Introduction

Three oncogenic viruses are known to cause tumors in chickens: MDV (Calnek and Witter, 1991), REV (Witter, 1991), and ALV (Payne and Purchase, 1991). MDV is a herpesvirus that causes malignant transformation of T cells in the chicken while REV and ALV are C-type retroviruses that can transform pre-B or pre-T cells (REV), B cells (ALV) or myeloid cells (the ALV subgroup J). The new virus, ALV-J, emerged lately as a widely distributed retrovirus in meat-type chickens (Payne, 1998). In turkeys, the following oncogenic viruses have been identified in tumor-bearing flocks: MDV, REV and lymphoproliferative disease virus (LPDV). In addition to their oncogenic ability, the viruses are also immunosuppressive, can cause growth retardation and may contaminate poultry vaccines.

The advantage of PCR application for the differential diagnosis of oncogenic viruses have been previously report-

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ed (Davidson *et al.*, 1995a,b, 1996, 1998). The survey of commercial tumor-bearing birds for avian oncogenic viruses indicated that both herpesviruses and retroviruses could infect the same bird under natural conditions. As dual infection was recognized as early as in 1967 (Cook *et al.*, 1967), we aimed to pursue the rate of multiple infections in field chickens and turkey flocks and to monitor systematically that rate in individual birds. That would indicate to what extent molecular interactions between viruses, as it was described in culture, might happen in nature in a similar way. The insertion of retrovirus sequences, and mainly the long terminal repeat (LTR) has been demonstrated to occur into MDV-1 genome (Isfort *et al.*, 1992). This, and other studies involving MDV (strain JM) or herpesvirus of turkeys (HVT) as the herpesvirus, and REV or ALV as the retrovirus, have been performed by co-cultivation of both viruses during 5–6 passages (Isfort *et al.*, 1992, 1994; Jones *et al.*, 1993, 1996; Kost *et al.*, 1993). Recently, Sakaguchi *et al.* (1997) and Endoh *et al.* (1998) reported the retrovirus LTR integration into MDV without co-cultivation of both viruses, but probably as a result of culture maintenance or the presence of avian endogenous viruses in the host cells. The MDV BC-1 strain at high passage in culture was found to contain several repeats of endogenous ALV RAV0 strain (Sakaguchi *et al.*, 1997). In other study the avian erythroblastosis virus (AEV), LTR was found to be integrated into the MDV prototype strain MD5 (Endoh *et al.*, 1998). Short REV LTR sequences (25–30 bp) were also recognized in the *Bam*H1-D, H and Q genomic fragments of several wild-type MDV-1 isolates (Isfort *et al.*, 1992). The authors suggest that the presence of such sequences might resemble ancestral insertions as the probability of a random sequence matching is small. These findings might also reflect the shuffling of genetic material between retroviruses and double-stranded DNA present in the host cell, either genomic or of herpesvirus origin (Brunovskis and Kung, 1996).

In the present study we aimed to broaden the knowledge on wild-type dual herpesvirus and retrovirus infection of the same host, so we initially determined the rate of multiple-infected flocks and birds in the field. We also report some data on the presence of short retroviral LTR sequences in the proximity of the 132 bp tandem repeat sequence of field MDVs.

## Materials and Methods

*Chicken flocks.* A total of 207 chicken and 52 turkey tumor-bearing flocks were surveyed over the period of five years (sporadically before 1994, and during the period of 1994 to June 1998). The study included various types of flocks of all ages. Between 3 and 6 live birds from each flock were bled using EDTA as anticoagulant, necropsied and the tumors were examined histologically. The plasmas were examined for REV antibodies.

*Oligonucleotide primers.* The PCR primers for MDV were selected to amplify the MDV-1 *Bam*H1-H 132 bp tandem repeats (Becker *et al.*, 1992). The primers for REV (Aly *et al.*, 1993) and ALV (provided by Dr. EJ Smith, ADOL, EL, USA) (Chen and Barker, 1984; Habel *et al.*, 1993) were designed to amplify the proviral LTRs. The rationale was based on the retrovirus ability of rapid integration into the host DNA with the exclusion of large parts of the provirus (Robinson and Gagnon, 1986). For the ALV J subgroup virus an additional primer set specific for the ALV-J env gene was used (Smith *et al.*, 1998). The primer sequences are shown below:

| | | |
|---|---|---|
| MDV-1: | direct (M1): | 5' TACTTCCTATATAGATTGAGACCGT |
| | reverse (M2): | 5' GAGATCCTCGTAAGGTGTAATATA |
| REV: | direct (R1): | 5' CATACTGGAGCCAATGGTT |
| | reverse (R2): | 5' AATGTTGTACCGAAGTACT |
| ALV: | direct (26): | 5' AAGTAAGGTGGTACGATCGTG |
| | reverse (30): | 5' CTGCTTCATTCAGGTGTTCGCAAT |
| ALV-J: | direct (H5): | 5' GGATGAGGTGACTAAGAAAG |
| | reverse (H7): | 5' CGAACCAAAGGTAACACACG |
| LPDV: | direct: | 5' CTTGCTGTTTTAAGCACAT |
| | reverse: | 5' AGCTATAGGCTCCGCGTCAA |

*DNA purification and PCR.* Blood DNA was purified with the Isocode Stix kit (Schleicher and Schuell) according to manufacturer's instructions and tumor DNA by the phenol/chloroform extraction method (Sambrook *et al.*, 1989).

The PCR for all systems except of the ALV-J *env* gene was as follows: DNA was amplified in a 25 µl PCR reaction with 10 mmol/l Tris HCl (pH 8.5), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 mmol/l dNTPs, primers (1.2 µmol/l, each) and 0.5 U of Taq polymerase (Advanced Biotechnologies Ltd). PCR cycling parameters were: one cycle of 94°C for 1 min, 31 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final elongation at 72°C for 10 mins.

The sequence homology of the ALV LTR and ALV-J (strain HPRS 103) LTR primers was analyzed and a difference of 21–23% was found; this value theoretically allows amplification. The ALV-J LTR (plasmid supplied kindly by M.A. Skinner, IAH,UK) was efficiently amplified with the ALV LTR primers.

PCR for the ALV-J env gene was performed according to Smith *et al.* (1998) and a 545 bp product was obtained: DNA was amplified in a 25 µl reaction with 10 mmol/l Tris-HCl (pH 8.8), 50 mmol/l KCl, 2.0 mmol/l $MgCl_2$, 400 µmol/l dNTPs, primers (1.2 µmol/l, each), 2% formamide and 0.5 U of Taq polymerase (Advanced Biotechnologies Ltd). PCR cycling parameters were: one cycle of 93°C for 1 min, 13 cycles of N°C for 1 min and 72°C for 1.5 min (with N starting at 60°C and decreasing by 1°C in each cycle), followed by 30 cycles of 93°C for 1 min, 48°C for 1 min and 72°C for 1.5 min, followed by a final elongation at 72°C for 10 mins.

*Sequencing and analysis of the PCR product.* The PCR products were separated in agarose gels and excised for purification using the Wizard PCR (Promega). The DNA was sequenced by the dye-terminator sequencing reaction in an Applied Biosystems Inc. (ABI) sequencer (Promega) using the R1 primer. The genomic sequence was aligned with those in the Gene Bank/EMBL using the Blast program (Blast Network Service, National Center for Biotechnology Information, NCBI).

**Table 6. Prevalence of multiple oncogenic virus sequences in blood and tumor DNA samples of commercial turkey flocks**

| PCR | Blood | | | | Tumor | | | |
|---|---|---|---|---|---|---|---|---|
| | Rate of multiple viral sequences in the same template according to: | | | | Rate of multiple viral sequences in the same template according to: | | | |
| | Total samples | | Positive samples (single+multiple) | | Total samples | | Positive samples (single+multiple) | |
| | No. | % | No. | % | No. | % | No. | % |
| MDV+REV | 2/58* | 3 | 2/22 | 9 | 0/24 | 0 | 0/14 | 0 |
| MDV+LPDV | 1/12 | 8 | 1/8 | 13 | 0/19 | 0 | 0/7 | 0 |
| MDV+REV+LPDV | 6/14 | 43 | 6/12 | 50 | NT | | NT | |
| Total | 9/84 | 11 | 9/42 | 21 | 0/43 | 0 | 0/21 | 0 |

*No. of positive/total flocks as deduced from the cumulative data from 3 assays.

**Table 7. Combined PCR analysis of DNA templates that carried both herpesvirus and retrovirus sequences**

| Heterologous primer combination | No. of DNAs yielding a chimeric PCR product | DNA origin | Approx. product size (bp) |
|---|---|---|---|
| M1+R1 | 0 | NA | NA |
| M1+R2 | 0 | NA | NA |
| M2+R1 | 3 | chicken | bird a: 900<br>bird b: 200<br>bird c: 200 |
| | 1 | turkey | bird a: 900, 700, 500 |
| M2+R2 | 0 | NA | NA |
| M1+L26 | 0 | NA | NA |
| M1+L30 | 2 | chicken | bird a: 1500,500<br>bird b: 150 |
| M2+L26 | 0 | NA | NA |
| M2+L30 | 7 | chicken | bird a: 900<br>bird b: 1000<br>bird c: 900<br>bird d: 1000<br>bird e: 1000<br>bird f: 1500<br>bird g: 1000 |

NA = not applicable; M1 = MDV forward; M2 = MDV reverse; R1 = REV LTR forward; R2 = REV LTR reverse; L26 = ALV LTR forward; L30 = ALV LTR reverse.

ond one. In the tumors, both sequences were detected in 16% and 20% of the 122 samples, after the first and second rounds amplification, respectively. Tables 3 and 6 show the cumulative data of these experiments. In the (MDV+REV)-positive chicken flocks 17% of the total blood and 20% of the total tumor DNAs had more than one virus in the same sample. When compared to the total PCR-positive templates (Table 2) with single or multiple viral sequences, these samples comprised 41% and 33% (blood and tumor, respectively). For the other categories the values varied from 20% to 44%. In summary, 14% and 17% of the total blood and tumor chicken DNAs carried multiple sequences, that is about 1/3 of the total positive samples. As the total number of turkey flocks was less than that of the chickens, less multiple viral sequence-positive DNA samples were detected, and that was restricted to the blood samples. While the rate of turkey blood DNA with MDV+REV was 3% compared to the total samples assayed, it was 9% compared to the positive samples. No multiple sequence-positive turkey tumor DNA was detected. When analysing the rate of DNAs with multiple sequences relating total samples of all flock categories, that was 3.87% (41/1057) of the chicken blood DNAs, 6.1% (47/766) of the chicken tumor DNAs and 3.9% (9/230) of the turkey blood DNAs.

*Molecular analysis of blood and tumor DNAs with both herpes and retrovirus sequences*

We analysed the retroviral LTR sequence presence at the proximal genomic regions of the MDV 132 tandem repeat sequence by the combined PCR (cPCR) approach. The method employed amplification of the same DNA template with six primer combinations including the homologous MDV (M1+M2) and retroviral LTR primers (R1+R2 for REV LTR and L26+L30 for ALV LTR) and four heterologous combinations of the two primer sets (M1+R1/L26, M1+R2/L30, M2+R1/L26 and M2+R2/30). Because the cPCR yield was low, in most cases a second round of PCR with the same primer combinations was performed. A total of 35 DNA templates from field birds that were PCR-positive for both MDV and retroviral LTR were analysed by cPCR; 20 of them were analysed with MDV and REV and 15 with MDV and ALV primers according to the initial flock diagnosis. In 13 DNAs the cPCR yielded chimeric products (Table 7). The most prevalent primer combinations that yielded chimeric PCR products were M2+R1 (with REV) (4 cases), M2+L30 (with ALV) (7 cases) and M1+L30 (with ALV) (2 cases, one of which reacted also with M2+L30). The controls were several DNAs from specific pathogen-free (SPF) chickens assayed with the 6 primer pairs, MDV+REV and MDV+ALV primer combinations, a cPCR-positive DNA

**Table 8. REV LTR homologous sequences found in chimeric PCR products**

| | |
|---|---|
| **A. Int. 1 sequence derived from a chicken tumor DNA** | |
| Int. 1 | TTNTCTAACGTGCAGCCT-GA |
| | ********** |
| REV-LTR | TTGTG-A-CGTGCGGCCCAGA |
| Homology: | 14/20 nt (70%) |
| **II. Int. b sequence derived from a turkey blood DNA** | |
| Int. b: | TACGCGCTANACTGA-CACANG |
| | ********** |
| REV-LTR: | TAAGCGCTATA-TAAGC-CAGG |
| Homology: | 15/21 nt (71%) |

with M2 primer to rule out possible random product amplification, and several DNAs from MDV-infected birds with MDV+REV and MDV+ALV primer combinations. No chimeric products were produced in any control, so we have deduced that the cPCR results were associated with the dual viral infection of the birds.

The chimeric products obtained with the M2 and R1 primers of two templates (one product from a chicken tumor DNA and 3 products from a turkey blood DNA) were sequenced using the R1 primer, and compared to the GeneBank database. The chicken tumor DNA product was homologous (67%) to the Chicken Repeat element 1 non-LTR type retrotransposon (CR1) (Burch *et al.*, 1993). In two of these products we detected 20-bp and 21-bp nucleotide stretches with a 70% and 71% homology to the REV-LTR (Table 8). Moreover, the amplification of the chimeric products using a retrovirus primer denotes that at least short nucleotide stretches homologous to retroviral LTR were present in field-originated MDVs. These stretches might resemble ancient retroviral insertions, as previously demonstrated (Isfort *et al.*, 1992). Accordingly, in the chicken product (Int.1) two 20-bp stretches were present (one homologous to the REV-LTR forward primer (R1) and the other shown in Table 8). In the turkey DNA four stretches could be identified: three homologous to the R1 primer, and one shown in Table 8 (Int. b).

## Discussion

In the present report we summarize the findings of a five-year survey on the causative oncogenic viruses of commercial chickens. Our aim was to evaluate the PCR efficacy in the differential diagnosis and to assess the prevalence of multiple virus infection in various commercial flocks affected by tumors. In the light of the present results it appears that the PCR is an efficient method for the differential diagnosis of avian oncogenic viruses, and especially, it is of a great value in the detection of infection by more than one virus, which is likely to occur in commercial flocks. We demonstrated that 25% (44/175) of commercial chickens and 24% (8/43) of turkey flocks carried multiple virus infections. Moreover, we showed that while during the years of 1994–1995 REV was the predominant retrovirus in chicken flocks, during 1997–1998 ALV was found to be the main retroviral counterpart. To our knowledge, our study is the first that determined systematically the rate of dual or triple virus prevalence in field birds.

As our purified DNAs originated from blood or tissue samples that included numerous cells, they represented heterogenous mixtures. We have to consider the possibility that part of the cells were infected with various viruses. There were differences between samples in the amount of MDV or proviral LTR; while some yielded MDV and REV PCR products in similar amounts, they differed in others; some had more MDV than retrovirus LTR and in others the opposite was detected. We faced the need to amplify twice some templates in order to obtain clear results.

The PCR cannot exclude the possible infection of the same cells in the sample with multiple viruses and the possible *in vivo* integration of retroviral sequences into MDV present in the same cell. The possibility that retroviral sequences might have been integrated into the MDV genome, as demonstrated *in vitro,* is still under investigation. We approached that issue by the cPCR using herpesvirus and retrovirus primers in heterologous combinations to identify chimeric PCR products that might depict both MDV and retrovirus sequences. As 1/3 of the samples (13/35) contained a sequence that allowed the chimeric product formation, and not all DNAs were positive, that indicated specificity. One of the sequenced chimeric products was homologous to the CR1 (Burch *et al.*, 1993). We still do not have evidence for a LTR or other retrovirus genomic fragment integration into MDV. However, the fact that the cPCR with one of the retroviral LTR primers resulted in the amplification of the fragment residing between it and the MDV primer denoted that a short stretch of nucleotides (20-25 nt) of the MDV genome was homologous to the retroviral LTR, on which these primers were based. Another two short nucleotide stretches (20-21 nt) were homologous (70–71%) to the REV-LTR. This result resembles the finding of Isfort *et al.* (1992) who demonstrated such a phenomenon in wild-type MDVs, and similarly to the previous study, these stretches resided in the same MDV genomic region.

To provide a broader characterization of multiple virus-infected DNA samples at the MDV molecular level and to determine the retrovirus status, we continue to analyze field MDVs with emphasis on other MDV genomic locations.

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