Contamination rate of Avian Leukosis viruses among commercial Marek's Disease vaccines in Assiut, Egypt market using Reverse Transcriptase-Polymerase Chain Reaction

Moemen A. Mohamed*, Tolba Y. Abd El-Motelib, Awad A. Ibrahim and Moustafa E. Saif El-Deen

Department of Poultry Diseases,
College of Veterinary Medicine, Assiut University, Assiut-Egypt
* Corresponding author

Abstract

Avian leukosis viruses (ALVs) in poultry may induce a variety of deleterious effects including tumors, increased mortalities, growth retardation and decrease in egg size and production that led to considerable economic losses. The identification of avian leukosis viruses (ALVs) in imported Marek's disease (MD) vaccines has raised concern about transmission of these retroviruses to vaccine recipients esp. poultry breeding stocks, so Egypt as one of importing countries requires freedom of infection with ALVs in such vaccines. Subgroup specific RT-PCR was undertaken on isolated RNA from 13 obtained commercial MD vaccines using six pairs of primers that correspond to envelope glycoprotein gene (gp85) which determines possible contamination with the six ALV subgroups: A, B, C, D, E, and J. The results indicated that RT-PCR assay for ALV-gp85 subgroup-E was positive for eight out of thirteen (61.5%) tested MD vaccines, while primers designed to detect subgroup A and J ALVs were positive for five out of thirteen (38.5%) and two out of thirteen (7.7%) respectively among examined vaccines. No ALVs was detected in 3/13 (23.07%) of commercially examined vaccines by using any of six primer pairs. Finally, the using of RT-PCR assay provides us a new, sensitive approach for identifying ALVs as a contaminant agent that will help greatly in applying this method for equipped labs as a quality control measure for testing delivered MD vaccines before its administration in poultry breeding stocks as well eradication programs through identifying infected birds.

Key words: Marek's disease, Vaccine contamination, Avian leukosis virus, RT-PCR

Abbreviations: ALV= avian leukosis virus; CEF= chicken embryo fibroblast; COFAL= complement fixation for avian leukosis; LTR= long terminal repeat; MDV=Marek's disease virus; RT-PCR=reverse transcriptase-polymerase chain reaction; REV=reticuloendotheliosis virus; SPF= specified-pathogen free.

Introduction

Avian leukosis viruses (ALVs) associated with a variety of neoplasms including lymphoid and myeloid leukosis. In commercial poultry flocks worldwide, ALVs are prevalent in several breeding flocks causing serious economic losses from tumor mortality in sexually mature chickens, carcass condemnation, growth rates, decreased egg production, and produce eggs of reduced size and quality (Gavora et al., 1980, 1982 and Payne and Fadly, 1997). Among the ALV-specific proteins, the envelope glycoprotein on the surface of the retroviral particles is the major subgroup specific determinant (Bova et al., 1991). Based upon differences in nucleotide sequences of their gp85 envelope gene, ALVs are divided into six subgroups. Subgroups A, B, C, D, and J are classified as exogenous viruses and can induce B-cell lymphomas in susceptible chickens (Bova and Swanstrom, 1987; Bova et al., 1988). Subgroup E ALV viruses are non-oncogenic endogenous viruses, present in nearly all chicken genomes, that are transmitted in a noninfectious form from one generation of chickens to the next in a Mendelian fashion along with the host genes (Calnek et al., 1991).

Congenital transmission accounts for the spread of ALV from one generation to the next, and the virus can also spread horizontally from chick to chick. To prevent transmission, the poultry industry must identify

infected eggs and chickens, and remove them from breeding populations. Because ALVs vertical transmission from dams to offspring, embryos from infected breeder hens and tissue 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 (Fadly and Payne, 2003). Further, chicken embryos and cells prepared from embryos obtained from an ALV-positive flock may harbor endogenous subgroup E ALV (Crittenden and Smith, 1984). Although ALV-E is not known to cause tumors in chickens, its presence in vaccines is not favorable, because it has a 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).

Many live virus poultry vaccines, including the vaccine against Marek's disease (MD), a T-cell lymphoma induced by an alpha herpes virus, are produced in chicken embryo fibroblasts (CEFs) obtained from specific-pathogen-free (SPF) chicken embryos (Witter and Schat, 2003).

Although current regulations are strictly observed and followed by vaccine manufacturers and suppliers of SPF embryonated eggs in many countries to be assured its produced biological products free from virus contaminants, accidental contamination of live virus vaccines of poultry with avian retroviruses, particularly REV, has been reported (Fadly, et al., 1996 and Takagi et al., 1996); previous reports of contamination of live virus poultry vaccines with ALV in Middle East and Egypt are rare, if any.

The complement fixation for avian leukosis (COFAL) test is routinely used by vaccine manufacturers to detect potential contamination with ALVs. It is conceivable that the COFAL test may be insensitive, on occasion to detecting ALVs, particularly if the contaminating viruses are present in extremely low titer or should the contaminating viruses replicate at a very low rate. Non-defective ALVs containing an endogenous long terminal repeat (LTR) are known to carry less transcription enhancer motifs within the U3 region, which may influence virus-induced transcription to result in slower replication and less oncogenesis compared with viruses carrying an exogenous LTR (Zavala and Cheng, 2006).

The polymerase chain reaction (PCR), in addition to its multitude of uses in molecular biology, is a valuable tool in the rapid and accurate diagnosis of many infectious agents (Smith et al., 1998). By utilising deoxyribonuclease (DNase) and reverse transcriptase, it can also identify the status of a retroviral infection by confirming the presence of either the retroviral RNA or its proviral DNA copy. Recently PCR was also used for the detection of vaccine contamination by ALV using primers targeting the variable envelope glycoprotein gene (gp85) that is the basis for dividing ALVs into their different subgroups A to E (Silva et al., 2007).

In light of the contamination of Marek's disease vaccines described herein, the objective of this study was to test commercial MD vaccines produced by manufacturers for contamination with ALV with the aid of RT-PCR using six pairs of primers for the specific detection of the members of ALVs subgroups.

Materials and Methods

MD vaccines: Several recently purchased commercial MD vaccines were examined. These vaccines representing 13 different serials and produced by two different manufacturers, A and B (A, 6 serials; and B, 7 serials), were received from vaccine manufacturer agents in Cairo-Egypt. Vaccine samples from manufacturer A and B contained serotypes 1 and 3 (CVI-998 and HVT) of MDV, or HVT and CVI-998 as monovalent vaccines contained one serotype either serotype 1 or 3. All vaccines were maintained in liquid nitrogen until assayed, with the exception of an overnight shipment in dry ice.

Primers Designing: For detection of avian leukosis viruses, six pairs of primers were synthesized at BioBasic Inc., Torbay Road, Markham Ontario, Canada. The primers were chosen according to published sequences of the endogenous and exogenous gp85env gene (Pham et al., 1999 and Smith et al., 1998) (Table 1). Detection of ALV in vaccines by using reverse transcriptase-polymerase chain reaction (RT-PCR).

Viral RNA extraction: Vaccines were first resuspended in 2ml RNAse free water. (Gibco, Grand Island, NY, USA). For RNA extraction, all samples were processed with the RNeasy® Mini kit, Cat. No. 74104 (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The protocol was modified as follows: initially 400 µl of sample supernatant was mixed with 300 µl of lysis buffer containing 6 µl ß-mercaptoethanol (Sigma-Aldrich Co., Germany). This mixture was incubated at room temperature for one hour. From this point on the manufacturer's instructions were followed.

RT-PCR: The QIAGEN OneStep RT-PCR Kit (QIAGEN Inc Valencia, CA 91355, USA) was used according to the manufacturer's instructions. Briefly, 10 µl of a 5× reaction buffer of OneStep RT-PCR, 2 µl dNTP mix, 2 µl OneStep RT-PCR enzyme mixture, 100 pmol of each oligonucleotide primers (Table 1), 0.25 µl RNAse inhibitor (40 U/µl, Promega) and 5 µl of RNA were mixed with RNase-free water to a final volume of 50 µl. All ingredients were kept on ice during handling.

Table-1. Primer sequences used in RT-PCR and PCR assays for detection of ALVs subgroups

ALV subgroup	Primer	Sequence (5'*-3')	Product Size	References
A	A-F A-R	GGCTTCAGGCCAAAAGGGGT GTGCATTGCCACAGCGGTACTG	232bp	Pham et al., 1999
B	B-F B-R	GGCTTTACCCCATACGATAG ACACATCCTGACAGATGGACCA	259 bp	Pham et al., 1999
D	D-F D-R	GGCTTCACCCCATACGGCAG CCATACGTCCTCACAGATAGAATA	258 bp	Pham et al., 1999
C	C-F C-R	TATTTCGCCCCAAGGGCCAC CCACGTCTCCACAGCGGTAAGT	238bp	Pham et al., 1999
E	E-F E-R	GGCTTCGCCCCACACTCCAA GCACATCTCCACAGGTGTAAAT	265bp	Pham et al., 1999
J	J-F J-R	GGATGAGGTGACTAAGAAAG CGAACCAAAGGTAACACACG	545bp	Smith et al., 1998

Negative controls consisted of nuclease-free water instead of RNA templates.

RT-PCR amplification profiles: The PCR reaction was performed in a Techne Cyclogen Thermocycler as follows: 30 minutes at 50°C (RT reaction); 94°C for 15 minutes (initial PCR activation); 39 three-step cycles of 94°C for 30 sec for denaturation, 58°C for 1 minute for annealing and 72°C for 2 minutes for extension; 72°C for 10 minutes (final extension). Since RT-PCR reactions were carried out overnight, an additional cycle at 4°C was also added to keep the RT-PCR product at a stabilizing temperature.

A 1 KB Plus DNA ladder, Cat. No. 10787-018 (Invitrogen Life Technologies, USA) served as molecular DNA size marker. Ten microlitres of RT-PCR and PCR products were analyzed by electrophoresis on 1% Nusieve agarose (Sigma Chemicals, Switzerland) with 2.5 mg/ml of ethidium bromide and 1X TAE buffer (20X) (Sigma, Switzerland) that is free of DNAase, RNAase, and proteinase. The gels were run at 70V: cm for 1.5 h and visualized using a UV transilluminator (Vilber Lourmat, France).

Reactions that produced the approximate expected size DNA fragment, as determined by the primer sets used in table (1), was considered positive. Samples that contained no amplification product, amplification product not of the expected size, or smears were classified as negative.

Results

RNA was extracted from 13 MD vaccines then examined with RT-PCR using specific primers that have the ability to amplify all subgroups of ALVs. Table 2 presents results of testing thirteen obtained commercial MD vaccines using RT-PCR

Primer set E-F and E-R designed to detect avian leukosis viral subgroup-E, amplified a product of approximately 265 base pairs in 8 of 13 (61.54%) tested vaccines (Table 2). However the presence ALV sub group A in the examined vaccines could be detected in 5 samples of 13 (38.46%) using primer set A-F and A-R (Table 2), that amplified a fragment of 246 bp. Primers J-F and J-R could amplify a fragment of 545 bp in 2 of 13 (15.4%) examined samples .

Specificity of the RT-PCR product was confirmed as no viral amplifications were detected in the samples containing no RNA or DNA templates respectively.

Discussion

Reports of contamination of live virus vaccines of poultry with ALV are rare esp. in Middle East and African countries. Reverse transcriptase activity was detected in all chicken-cell-derived measles and mumps vaccines, suggesting the presence of endogenous ALV elements (Tsang et al., 1999 and Hussain et al., 2001). So, it is important to assure that live virus vaccines of poultry or humans that are produced from chicken-originated ingredients be free from both avian retroviruses, namely REV and ALV.

Avian retroviruses can be transmitted vertically from dams to offspring, embryos and tissue cultures prepared from such embryos may harbor such viruses (Fadly and Payne, 2003). Therefore, embryos or cells prepared from infected embryos could serve as a source of retrovirus contamination of poultry and of other vaccines produced from such ingredients. Accidental contamination of live virus poultry vaccines such as MD virus vaccines with reticuloendotheliosis virus (Takagi et al., 1996 and Reimann and Werner 1996) is an important source of virus infection and can cause runting disease as well as lymphomas in vaccinated chickens, resulting in significant economic consequences.

Our results as shown previously in table 2 and Fig. (1-4) clearly indicate that the examined commercial MD vaccines produced by two companies were contaminated with an exogenous and endogenous ALV. Furthermore, the results of characterizing the

Table -2. ALVs subgroups isolated from commercial MD vaccines using subgroup specific RT-PCR.

Number of Sample	MD vaccine strain	Manufacturer Source	Isolated ALVs Subgroups					
			A	B	C	D	E	J
1.	HVT+ CVI-988	A	+	-	-	-	+	-
2.	CVI-988+FC126	A	-	-	-	-	+	-
3.	CVI-988+FC126	A	+	-	-	-	+	-
4.	CVI-988	A	-	-	-	-	-	-
5.	CVI-988	A	-	-	-	-	-	-
6.	HVT	A	-	-	-	-	-	+
7.	CVI-988+HVT	B	+	-	-	-	+	-
8.	CVI-988+HVT	B	-	-	-	-	+	+
9.	CVI-988+HVT	B	+	-	-	-	-	-
10.	CVI-988	B	-	-	-	-	+	-
11.	CVI-988	B	-	-	-	-	+	-
12.	HVT	B	+	-	-	-	+	-
13.	HVT	B	-	-	-	-	-	-

contaminant ALVs using RT-PCR assay could identify ALV subgroup A among 5 examined vaccines as well as ALV subgroup E among 8 tested samples and ALV subgroup J contaminant in two samples out of 13 tested. The presence of retroviruses with Marek's disease vaccines as mentioned in our results may represent a deleterious finding due to co-infection with such two viruses (MDV and ALV) in cells may promote replication and pathogenesis of one or both virus types (Pulaski et al., 1992). For example, serotype 2 MDV strains may enhance expression of bursal lymphomas in dually infected chickens partially as a result of enhanced retroviral gene expression (Marsh et al., 1995 and Aly et al., 1996). Thus, contamination of any vaccine contaminated with ALV and containing serotype 2 MDV could potentially result in a higher incidence of ALV-related lymphomas.

In spite of quality control that be followed in companies for producing vaccines free from extraneous agents stated in the European Pharmacaopoeia (1995) and in the Code of Federal Regulations (1996), our findings could be explained for a variety of reasons; firstly, the Complement-Fixation-Test for Avian Leukosis (COFAL) has been in service and used effectively to accomplish this purpose (Sandelin and Estola, 1975) but a recent reason being that the viruses involved do not seem to propagate to high titers initially, in particular when cells are coinfected with MDV and ALV. Furthermore, the presumed presence of an endogenous LTR may hinder the ability of the contaminating ALVs to propagate to detectable virus titers on first attempt by COFAL test (Ruddell, 1995). Secondly, SPF eggs and chicken embryo fibroblasts used to produce the vaccines were likely the source of ALV contamination (Zavala and Cheng, 2006). Thirdly, the presence of ALV subgroup J as contaminant in our findings may be due to spontaneous emergence of ALVs by way of recombination is an additional possibility that could result in flock infection and vaccine contamination. This mechanism has been suggested as instrumental for the emergence of viruses such as ALV-J (Benson et al., 1998).

Finally, more extensive studies are needed to assess the risk of vaccine infection with the contaminating ALVs in commercial chickens to determine the oncogenicity degree of detected ALVs. Also, this study demonstrates that RT-PCR for the detection of ALVs contamination in viral poultry vaccines is reliable and sensitive that could be routinely used for viral safety testing of vaccine batches before its use and can be considered a useful technique in diagnosis.

Acknowledgements

The Department of Poultry Diseases Department, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt is thanked for a grant supporting this work.

References

1. Aly, M., Witter, R., Fadly, A., (1996): Enhancement of reticuloendotheliosis virus-induced bursal lymphomas by serotype 2 Marek's disease virus. *Avian Pathology* 25:81–94.
2. Benson, S., (1998): Independent isolates of the emerging subgroup avian leukosis virus derive from a common ancestor. *Journal of Virology* 72: 10301–10304.
3. Bova, C., Swanstrom, R., (1987): Host range determinants of avian retrovirus envelope genes. Positive Strand RNA Viruses. Alan R. Liss, New York, pp. 509–518.
4. Bova, C., Olsen, J., Swanstrom, R., (1988): The avian retrovirus en6 gene family: molecular analysis of host range and antigenic variants. Journal of Virology 62, 75–83.
5. Bova, C., Olsen, J., Swanstrom, R., (1991): Genetic analysis of the Rous sarcoma virus subgroup D en6

gene: mammal tropism correlates with the temperature sensitivity of gp85. Journal of Virology 65, 2073–2080.
6. Calnek, B., et.al. (1991): Diseases of Poultry, 9th edn. Iowa State University Press, Ames, IA.
7. Crittenden, L., Smith, E., (1984): A comparison of test materials for differentiating avian leukosis virus subgroup specific antigens of exogenous and endogenous origin. Avian Disease 28, 1057–1062.
8. Crittenden, L., et.al. (1987): Embryonic infection with the endogenous avian leukosis virus Rous associated virus-0 alters response to exogenous avian leukosis virus infection. Journal of Virology 61, 722–725.
9. Fadly, A., et.al.(1996): An outbreak of lymphomas in commercial broiler breeder chickens vaccinated with a fowl pox vaccine contaminated with reticuloendotheliosis virus. Avian Pathology 25, 35–47.
10. Fadly, A., Payne, L., (2003): Leukosis/sarcoma group. In: Diseases of poultry, 11th ed. Y. M. Saif, et.al.eds. Iowa State University Press, Ames, IA. pp. 465–516.
11. Gavora, J., et.al.(1980): Lymphoid leukosis virus infection. Effects on production and mortality and consequences in selection for high egg production. Poultry Science 59, 2165–2178.
12. Gavora, J., et.al.(1982): Performance of meat-type chickens test-positive and -negative for lymphoid leukosis virus infection. Avian Pathology 11, 29–38.
13. Hussain, A., et.al.(2001): Lack of evidence of endogenous avian leukosis virus and endogenous avian retrovirus transmission to measles, mumps and rubella vaccine recipients. Emerging Infectious Dis. 7: 66–72.
14. Marsh, J., Bacon, L., Fadly, A., (1995): Effect of serotype 2 and 3 Marek's disease vaccines on the development of avian leukosis virusinduced pre-neoplastic bursal follicles. Avian Diseases 39: 743–751.
15. Payne, L., Fadly, A., (1997): Leukosis:sarcoma group. In: Calnek, B., Barnes, H., Beard, C., McDougald, L., Saif, Y. (Eds.), Diseases of Poultry, 10th Edition. Iowa State University Press, Ames, IA, pp. 414–466.
16. Pham, T., Spencer, J., Johnson, E., (1999): Detection of avian leukosis virus in albumen of chicken eggs using reverse transcription polymerase chain reaction. *Journal of Virological Methods* 78: 1–11.
17. Pulaski, J., Tieber, V., Coussens, P., (1992): Marek's disease virus-mediated enhancement of avian leukosis virus gene expression and virus production. *Virology* 186: 113–122.
18. Reimann, I., Werner, O., (1996): Use of polymerase chain reaction for the detection of reticuloendotheliosis virus in Marek's disease vaccines and chicken tissues. *The Journal of Veterinary Medical Science* 43:75–84.
19. Ruddell, A., (1995): Transcription regulatory elements of the avian retroviral long terminal repeat. *Virology* 206:1–7.
20. Sandelin, K., Estola, T., (1975): Testing and management of a specific pathogen free chicken flock with special reference to avian leukosis virus infections. *Acta Veterinaria Scandinavica.* 16: 341–356.
21. Silva, R., Fadly, A., Taylor, S., (2007): Development of a Polymerase Chain Reaction to Differentiate Avian Leukosis Virus (ALV) Subgroups: Detection of an ALV Contaminant in Commercial Marek's Disease Vaccines. *Avian Diseases* 51: 663-667.
22. Smith, L., et.al.(1998): Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. *Virus Research* 54, 87–98.
23. Takagi, M., et.al.(1996): Detection of contamination of vaccines with the reticuloendotheliosis virus by reverse transcriptase polymerase chain reaction (RT-PCR). *Virus Research* 40: 113–121.
24. Tsang, S., et.al.(1999): Evidence of avian leukosis virus subgroup E and endogenous avian virus in measles and mumps vaccines derived from chicken cell: investigation of transmission to vaccine recipients. *J. Virol.* 73: 5843–5851.
25. Witter, R., Schat , K., (2003): Marek's disease. In: Diseases of Poultry, 11th ed. Y. M. Saif, H. J. Barnes, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne, eds. Iowa State University Press, Ames, IA. pp. 407–465.
26. Zavala, G., Cheng, S., (2006): Experimental Infection with Avian Leukosis Virus Isolated from Marek's Disease Vaccines. Avian Diseases 50, 232–237.

****