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Improved quality control protocol for fowl pox virus vaccines based on the use of PCR and sequence analysis to detect REV and ALV as contaminants

Radwan, A.A*; Hussein, HA**; El-Ibiary*, E. A.; El-Safty*, M.D.; El-Sabagh**, I. M and El-Deeb, A.H**

*Central Laboratory of quality control for Evaluation of Veterinary Biologics, Abbassia, Cairo, Egypt
**Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza-Egypt, 12211

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SUMMARY

In the present study, twenty one Fowl pox virus (FPV) vaccines were collected in the period between 2003-2005 and tested using the currently used and an improved quality control protocols. Results of currently used quality control protocol revealed negativity of all tested vaccines for the presence of contaminants and the results were satisfactory. Using PCR to detect Reticuloendotheliosis virus (REV) as contaminant in such vaccines revealed negative result except one suspected contaminated vaccine. Inoculation of this vaccine in egg, chicken embryo fibroblasts (CEFs), Specific pathogen free (SPF) chicks for three passages and testing of samples collected from inoculated host revealed positive amplification using REV specific primers. Sequence analysis of the obtained amplification fragment for REV revealed its negativity and confirmed the non specific amplification of such primers which were previously published in several PCR studies for REV. Using avian leucosis virus (ALV) sets of primers to detect groups A , B , C, D and J in a PCR reaction revealed positive amplification of ALV fragment and confirming the contamination of tested vaccine with ALV. The study proposes the importance of using PCR followed by sequence analysis of the amplified product to confirm the contaminations found in the FPV vaccines.

INTRODUCTION

Fowl pox (FP) is one of the oldest known viral diseases affecting susceptible chickens of all ages (Tripathy and Reed, 1997). The disease occurs either in a mild form with focal skin lesions or in a severe

form in which generalized lesions appears in any part of the body with respiratory distress (Tripathy and Reed, 1997). In layers, egg production is impaired and in young birds the growth is retarded (Beard et al., 1991). Recently, the disease become under control by vaccination and because of improvement in the management of practices and hygienic condition. In 1920, it was recognized that vaccination was an effective means of controlling the disease and the immunity could be established only when the virus isolive and that the cutaneous route was the most effective method of vaccination (De-Bliek and Heelsbergen, 1923).

Reticuloendotheliosis virus (REV) is an avian oncornavirus that is antigenically and structurally unrelated to viruses of leucosis/sarcoma group (Witter, 1991). REV is associated with runting syndrome, high mortality, immunosuppression and neoplasia associated with T and/or B cell lymphomas in domestic poultry and other avian species (Witter, 1991 and Filardo et al., 1994). The REV proviral DNA contains two identical long terminal repeats (LTR) and a complete set of genes including group-specific antigen (gag), protease (pro), polymerase (pol), and envelope (env). The LTR was the most divergent region, exhibiting various deletions and insertions (Barbosa, et al., 2007).

Avian Leucosis Virus (ALV) was the most common naturally occurring retrovirus associated with neoplastic disease conditions in domestic poultry. ALV could be transmitted from one chicken to another by inoculation of cell-free filtrates derived from tumor tissues obtained from diseased birds (Fadly et al., 1996). Retrovirus replication; is unique and complex; starts with reverse transcription of virion RNA into double-stranded DNA by the reverse transcriptase. These linear double-stranded DNA intermediates are circularized, integrated into the host chromosomal DNA, and then used for transcription, including the transcription of full-length genomic RNA and various mRNAs (Murphy et al., 1999).

REV is considered a potential hazard in case of using chicken embryo and cells for preparation of vaccines. REV had been detected and isolated from chickens suffering from high mortalities with nervous manifestation and feathering abnormalities after vaccination with Marek`s disease virus (MDV) vaccines at one day old (Yuasa et al., 1976 and Jackson et al., 1977). Also, Fadly et al., (1996) recorded an outbreak of lymphomas in broiler breeder flocks following vaccination with fowl pox virus (FPV) vaccines contaminated with REV. FPV has a long genome so it may integrated with by another virus such as REV. Integration of REV into the genome of FPV of field isolates and vaccine strains has been reported (Diallo et al., 1998; Moore et. al, 2000, Garcia et al., 2003 and Singh et al., 2003 and 2005).This integration was screened by polymerase chain reaction (PCR) for the presence of REV in the

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DNAs of nine avian pox viruses (Kim and Tripathy, 2001).

The present study started in 2003; the protocols used for quality control of FPV vaccines at that time did not include the PCR for detection of contaminants in poultry vaccines. Therefore, the aim of the study was the improvement of the quality control protocol for FPV vaccines based on the use of PCR followed by sequence analysis of the amplified products to detect viral contaminants with special emphasis on REV and ALV.

Material and Methods:

Vaccine samples:

Twenty one live FPV vaccines from different companies were collected in the period between 2003-2005.

Table (1): Detailed Features and Data of the tested vaccines

Sample No.	Seed virus	Company	Expiry Date
1	WB	Intervet	12/2004
2	Cutter strain	schering	11/2004
3	Cutter strain	Schering	04/2005
4	Brescia/P1	Izo	04/2006
5	Cutter strain	Mbl	03/2006
6	WB	Intervet	01/2006
7	WB	Intervet	09/2006
8	DCEP-25	Merial	12/2005
9	DCEP-25	Merial	03/2006
10	WB	Intervet	11/2006
11	WB	Intervet	06/2006
12	Cutter strain	Schering	12/2004
13	Cutter strain	Biomune	05/2005
14	Cutter strain	Biomune	02/2006
15	Cutter strain	Biomune	12/2004
16	WB	Intervet	07/2006
17	Baudette	Pox dept.	06/2006
18	Baudette	Pox Depet	10/2006
19	Cutter strain	Schering	05/2006
20	DCEP-25	Neuva	10/2005
21	DCEP-25	Neuva	03/2004

Detection of Extraneous viral pathogens by the chicken embryo inoculation: The tested vaccines were resuspended as recommended by manufacturers then one volume of prepared vaccines was mixed with nine volumes of the FPV antiserum to neutralize the virus. After neutralization, 0.1 ml of the vaccine–serum mixture was inoculated in the yolk sac, allantoic and on the chorioallantoic membrane (CAM) of SPF embryonated chicken eggs (ECEs) then incubated with daily observation for 7 days. All embryos and CAMs from embryos which die after the first

day were examined (US Code of Federal regulations, 1989).

Detection of haemagglutinating viruses: The assay was performed according to the standard protocols (US Code of Federal regulations, 1989). After resuspension and neutralization as mentioned before, the vaccine-serum mixture is diluted by 30 ml of sterile diluent/1000 doses and used as inoculum. 0.2 ml of the diluted inoculums was inoculated into allantoic cavity of SPF eggs then incubated with daily observation for 5 days. After incubation, the allantoic fluid from each egg was tested separately for haemagglutinating activity.

Detection of Extraneous viral pathogens by tissue culture inoculation: After neutralization of vaccine with antiserum, 0.1 ml of the mixture contains 10 doses of tested vaccines was incubated in 10 Petri dishes containing 10^6 CEF cells and incubated at 37 °C for 1 hour. After adsorption, 5ml fresh medium were added per dish then incubated for at least 7 days at 37 °C. A second passage was carried out and the cultures were examined to detect any cytopathic effect (CPE) or heamadsorbent agent **(US Code of Federal regulations, 1989)**.

Detection of REV using ELISA:

Solid-phase ELISA was carried out according to US Code of Federal regulations, 1989. Briefly, ELISA plates were coated with mixture of 11A25 and 11C237 REV monoclonal antibodies then blocked with 5% non-fat dry milk in PBS. 100µl of tested material (CEF cells inoculated by FPV vaccine) were dispensed to wells and incubated in the plates for 1 hour at 37°C. After washing, the plate was incubated for 1 hour at 37°C with rabbit anti-REV followed by three cycles of washing. The plates were then incubated with peroxidase conjugated goat anti-rabbit IgG diluted 1/1000 followed by three cycles of washing. The reaction was developed with H_2O_2/ ABTS. Further color development was stopped by SDS (0.5%) then the plate was read at 405nm wavelength.

Detection of REV as a contaminant in FPV vaccine using PCR: The DNA of FPV was extracted using GenisolTM Maxi-prep kit [AB gene] which has been formulated for rapid isolation of high molecular weight DNA. The primers sequences for PCR are as follow: Forward REV primer: 5̀-CAT ACT GGA GCC AAT GGT T-3̀ and Reverse REV primer: 5̀-AAT GTT GTA GCG AAG TRA T-3̀ (Davidson et al., 1995). PCR assay was performed using 10 µm of each forward and reverse primers in PCR reaction containing 2x Reddy Mix Master mix buffer optimized for PCR, which included a dye and precipitant to facilitate gel loading, dNTP mix (0.2 mM each), thermoprime puls DNA polymerase (1.25 u/50 µl) and MgCl2 (1.5 mM) [AB gene]. The PCR cycling profile consisted of one cycle at 94°C for 2 min for initial denaturation, and then 35 amplification cycles, with each cycle consists of 94°C for 45 sec (denaturation), 55°C for 45 sec

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(annealing) and 72°C for 45 min (extension) followed by a final extension cycle of 5 min at 72°C. The PCR products were analyzed on 1.25% agarose gel containing 0.5 µg/ml ethidium bromide. The assay was carried out directly on vaccine vials and after inoculation in SPF-ECEs (CAM), CEF cells and SPF chicks (blood).

Sequencing of the obtained REV PCR band: The PCR products of the amplification assay using REV primers was cut from the agarose gel and gel slices containing DNA bands were purified using montage DNA gel extraction kit (Millipore). The eluted DNA was sent for sequencing at the sequencing unit in VACSERA, Giza, Egypt.

Detection of ALV in suspected positive REV contaminated vaccine using PCR: The assay was carried out on the FPV DNA that previously extracted from vaccine vials as well as from ECEs and CEF cells inoculated with vaccine. The primers sequences for PCR are as follow: AD1:5'-GGG AGG TGG CTG ACT GTG T-3', H5:5'-GGA TGA GGT GAC TAA GAA AG-3', H7b:5'-GAA CCA AAG GTA ACA CAC GT-3' and CAP-A: 5'-AGA GAA AGA GGG GYG TCT AAG GAG A-3' (Hussein et al., 2006). The H5 + AD1 primers are used to detect ALV sub-groups A-E with expected band size of 360 bp, H5 + CAP-A primers are used to detect ALV sub-groups A with expected band size of 694 bp and H5 + H7b primers are used to detect ALV sub-groups J with expected band size of 544 bp. The PCR mix and cycling profile is similar to that used with REV except the annealing temperature was performed at 58°C.

RESULTS

Extraneous viral pathogens detection by the chicken embryo inoculation: All harvested CAMs from the inoculated eggs by the tested vaccines did not show any pock lesions after neutralization of the vaccines with the specific anti-pox virus antiserum. Figure (1) show the difference between the CAMs collected from inoculated eggs before and after neutralization.

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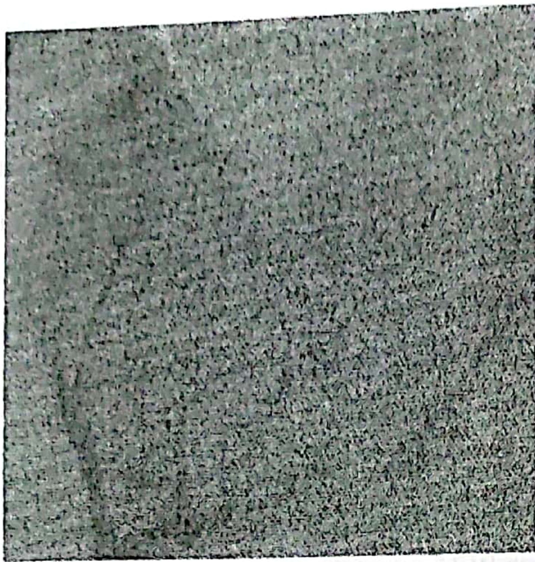

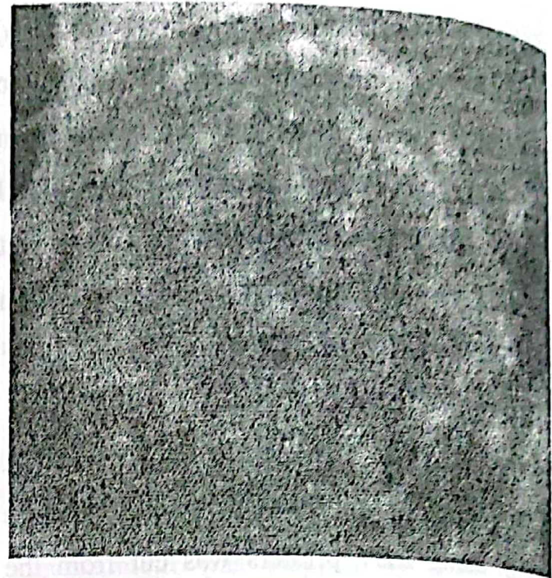

Figure (1): Macroscopical feature of CAMs showing the characteristic pock lesion of FPV before neutralization with specific antiserum in comparison with normal membrane after neutralization.

Detection of haemagglutinating viruses: All tested vaccines were negative for the presence of any contaminating haemagglutinating agents after examining the allantoic fluid of the inoculated eggs for haemagglutinating activity.

Detection of Extraneous viral pathogens by tissue culture inoculation: After inoculation 10 Petri dishes containing CEF cells per each vaccine sample with 0.1 ml of vaccine serum mixture. There was no specific CPE observed on the inoculated tissue culture cells. Also, examination of the inoculated cells for any haemadsorption activity after treatment with 0.5% RBCs suspension showed negative haemadsorption activity.

Detection of REV using ELISA: All tested FPV vaccines found to be negative for REV contamination in ELISA test when compared with positive and negative control.

Detection of REV as a contaminant in FPV vaccine using PCR: The PCR assay revealed that all tested FPV vaccines gave negative result when the assay was performed on vaccines vials and after inoculation on CEF cell culture (Figure 2A). Whereas, after inoculation of vaccines in ECEs and 1 week old SPF chicks the assay revealed that all tested vaccines gave negative results except one vaccine was suspected to be positive as it revealed band with approximately 200 bp (figure 2B)(the expected REV amplified band size was 291bp when reference REV DNA was used).

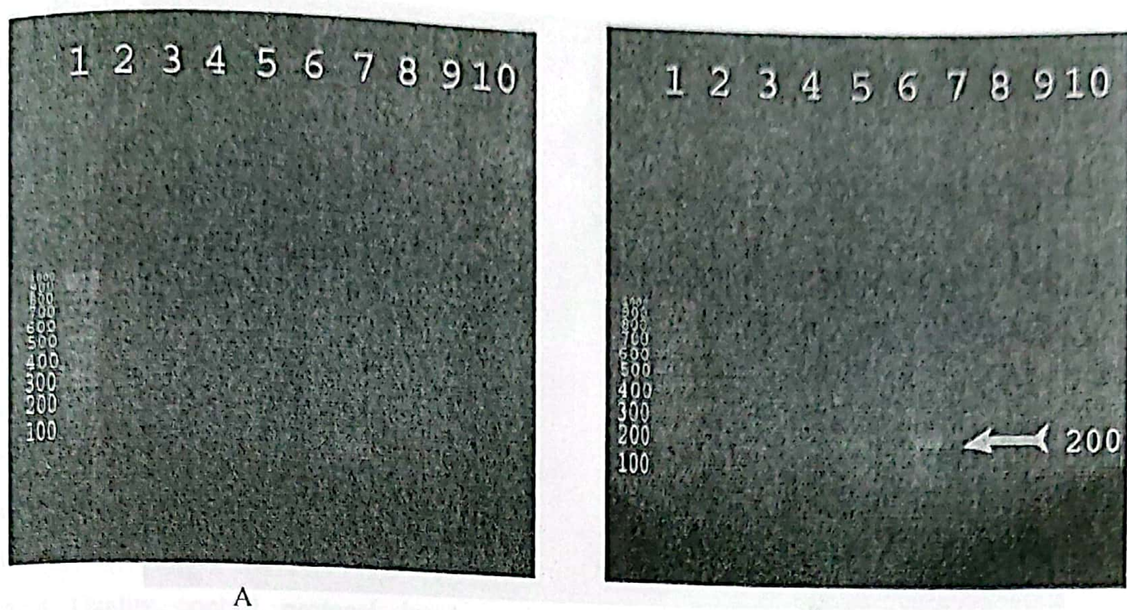

Figure (2): Electrophoresis of PCR for detection of REV contaminant in FPV vaccines alone with 100 bp PCR marker. A- Demonstrate the PCR assay on vaccine vials and after inoculation on CEF cells. B- Show suspected positive bands at 200bp after inoculation of vaccines in ECEs and SPF chicks.

Sequencing of the REV suspected positive band:

The purified DNA of REV suspected band (200bp) was sequenced in VACSERA sequencing unit. The obtained nucleotide sequences are shown in the following box. The obtained sequence was analyzed using computer software (BLAST) via the internet (www.ncbi.nlh.gov.) which revealed that the nucleotide sequence was non specific and did not related to REV.

GTGGCTCGGCATCCTGGCGGCTTCTGCTGGNGAAAGNAAA
GCCCGTTTCCCCCGCCCGAGAGAGCGCTTGCAGTTCGGAC
ACACCGTGTTTCCGAGCGAC TTGTGCGGAC TGTCGGTACA
ACATTAATACAACNTTAA

PCR testing of the suspected FPV vaccine for ALV contaminant:

Testing of DNA extracting from the vaccine vial, inoculated ECE and inoculated CEF cells by PCR using primers specific for ALV subgroups A to E, subgroup A and subgroup J revealed strong positive bands of corrected expected size 360 bp (ALV sub-group A-E) in the DNA of the vaccine vial and the harvests of the inoculated eggs. While, the harvested CEF cells shown faint band indicate the possibility of presence of endogenous ALV figure (3).

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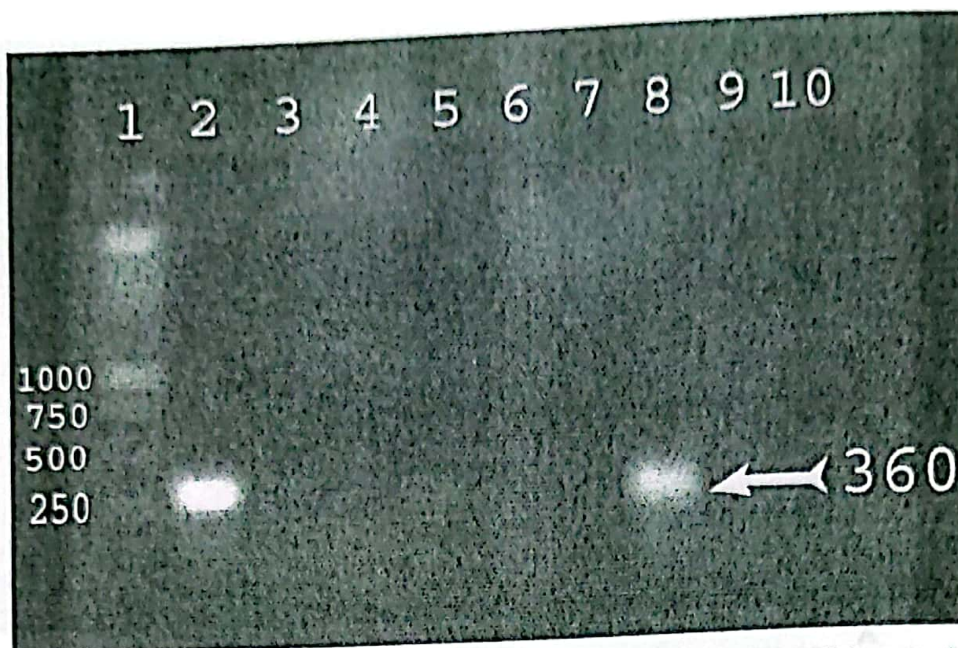

Figure (3): Detection of ALV in DNA extracted from vaccine vial, CEF and ECE harvests after inoculation with the FPV vaccine. Lane 1 PCR marker, Lanes 2, 5 and 8 amplified bands using specific primer to subgroups A-E for DNA extracted from vaccine, harvests of inoculated CEF and ECE, respectively. Lanes 3, 6 and 9 PCR products using specific primer to subgroup A for DNA extracted from vaccine, harvests of inoculated CEF and ECE. Lanes 4, 7 and 10 PCR products using specific primer to subgroup J for DNA extracted from vaccine, harvests of inoculated CEF and ECE.

DISCUSSION

In the present study an improved protocol for quality control of FPV vaccines was adapted to be capable for detection of REV as a contaminant in the end product of FPV vaccines. To fulfill the goal of the study, twenty one FPV vaccines were used. FPV vaccines have been routinely used for more than half a century to virus infection in commercial poultry in areas where the disease is endemic. However, in recent years, outbreaks of FPV have occurred in previously vaccinated flocks (Nebraska (1992), New York (1997), Oklahoma (1997) and California (1998), USA). A possible explanation for this problem is the emergence of variant strains of FPV or not mutually exclusive, postulate is that the novel FPV exhibit enhanced virulence due to the integration of REV into their genomes (Singh et al., 2000).The presence of integrated REV provirus in FPV genome resulted in inability of current vaccines to induce adequate immunity in poultry (Singh et al., 2005; Wang-JiaNing et al., 2006).

Application of PCR in detection of REV has been previously used (Fadly and Witter, 1997). PCR assay has been offered an increase sensitivity of assays used to detect REV in vaccines (Aly et al., 1993). The PCR

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assay designed to amplify the long terminal repeat (LTR) region of REV identified REV LTRs in many of the commercial FPV vaccines evaluated. These commercial vaccines were not thought to be contaminated with replicating REV because of the lack of REV outbreaks, the lack of in vitro amplification, and lack of a serological response to REV (Aly et al., 1993).Until recently, routine testing of live virus poultry vaccines for contamination with REV can not detect contamination of FPV vaccines. As the current. Quality control protocol involves detection of viral pathogens by the chicken embryo inoculation test, detection of viral pathogen by tissue culture inoculation, detection of REV using ELISA. Recently and after the proposal of the current study in 2003, PCR was added to the current protocol for quality control.

The present study proposes an improved protocol of quality control of FPV vaccine to insure the detection of the integrated REV sequences in the FPV genome. The improved protocol was based on the use of PCR to detect the contaminating REV in FPV vaccines. After inoculation of the FPV vaccines in SPF ECE, CAMs harvested from the inoculated eggs and were tested for REV by PCR and the results proved the efficiency of PCR to detect the REV genome region in the extracted DNA from the membranes when compared with the tested non inoculated control membranes. The results revealed that there is one suspected to be contaminated vaccine (figure 2B). PCR increased the sensitivity of biological assays used to detect REV in the tested vaccines. This has been previously repeated sensitive and specific method for the detection of REV infection (Aly et al., 1993).

Fowl pox virus vaccines in the study were inoculated in CEF cell culture for three successive passages. The cell were harvested after 6th day for use in the PCR on the extracted DNA from the first and third passages the results revealed negative for all vaccines samples. Using cell culture in virus propagation and application of PCR to detect REV in the harvested cells were reported by others (Ramos et al., 2002 , Tadese and Reed , 2003 and Duan-Hong An et al., 1999).The results in the present study were negative in cell culture. This may be due to the tested FPV vaccines are mainly egg-adapted and might need more passages to develop visible changes in cell culture. Also, there was no observed CPE in the inoculated cells. Indeed, the use of cell culture in the quality control to detect REV is questionable and need further studies.

We anticipate that the PCR is more sensitive than ELISA in detection of REV in the inoculated CEF culture. Moreover, the concentration of REV in contaminated vaccines may be critical and influence the sensitivity of the test used. Failure of ELISA to detect REV in inoculated CEF culture by 6 days PI suggests that ELISA may not be the

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test of choice for testing CEFs inoculated with contaminated FPV vaccines (Fadly and Witter, 1997).Because CPE in cell cultures may not be seen on primary isolation, the presence of REV is confirmed by PCR .The PCR assay was designed to amplifies the 291 base pairs product of REV LTR and has been shown to be a sensitive and specific method for detection of various strains of REV in infected CEFs and also in the blood of SPF chickens inoculated with contaminated FPV vaccines. Further, REV was detected in the pock lesion of SPF eggs resulted from inoculation of SPF egg by contaminated FPV vaccines. Recently, using PCR tests that amplify REV envelope and REV 3' LTR sequences provided a more accurate assessment of the insertion of REV provirus in FPV than PCR assays that amplify the REV 5' LTR (Fadly and Garcia , 2006). Such discrepancies in the PCR specificity was observed in the present study as the amplified product was of 200bp whereas from the reference was 291bp.

One vaccine suspected to be positive for contamination when tested in SPF chicks. To confirm such positive results, the vaccine was inoculated into SPF chickens and the REV was detected after three weeks post vaccination by PCR. Application of PCR on the collected blood samples of the inoculated SPF chick revealed that the suspected band of REV was detected at third week in the tested group compared with the control non-vaccinated group. Also, neutralizing the vaccines with specific anti-FPV antisera and application of PCR on samples of the inoculated chicks with mixture revealed that REV was negative by PCR in all collected blood samples of this group. This indicates the integration of REV into the FPV genome. Thus the present study demonstrates the successful use of PCR in the quality control protocol to detect the contaminating REV. Unfortunately, sequence analysis for the suspected band revealed that the obtained band did not related to REV. therefore, this result led us to test this vaccine for ALV contamination.

PCR for ALV was developed for the detection of contamination of vaccines produced in embryonated eggs and cell cultures derived from chicken. Viral RNA of all 5 subgroups (A-E) was isolated and amplified using specific primers. This system provides a rapid and specific in vitro method for the detection of ALV RNA as a contaminant and may be applied for quality control of avian vaccines (Haptli, et al., 1997).

ALV infection in the progeny is of major problem in the eradication program adapted by most of the major chicken producing companies. The presence of ALV in the eggs used for production of FPV vaccines is expected when these eggs were obtained from commercial and not SPF eggs. The 21 FPV vaccines tested in the present

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study were not tested for ALV contamination except one suspected vaccine which revealed non specific PCR amplification when tested by PCR using REV specific primers. This led us to test for ALV contamination which reveals positive amplification. The primers used in ALV-PCR were specific for subgroups A to E and the amplified fragment need to be sequenced in future studies to confirm which the subgroup specially the exogenous and not endogenous one.

Indeed, the use of PCR followed by sequence analysis is highly recommended in the study. Moreover, the study propose an improved and adapted protocol for quality control protocol of the FPV vaccines based on the use of PCR followed by sequence analysis of the obtained fragment.

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برتوكول محسن لرقابة جودة لقاحات جدري الطيور بالإعتماد على استخدام تفاعل البلمرة المتسلسل وتحليل التتابع النيوكليوتيدي للكشف عن فيروس الرتيكيلواندوثليوزيس وليكوزيس الدجاج كملوثات

*أمنية رضوان ، **حسين على حسين ، * إلهام الإبياري ، * منير الصفتي ، **إبراهيم محمد الصباغ ، **أيمن هانئ الديب

*المعمل المركزي للرقابة على المستحضرات الطبية البيطرية – العباسية
**قسم الفيروسات – كلية الطب البيطري – جامعة القاهرة

في هذه الدراسة تم تجميع عدد (21) عينة لقاح جدري الطيور في الفترة بين عامي 2003- 2005 واختبارهم باستخدام البروتوكولات المتبعة حاليا ، وكذلك البروتوكولات المحسنة.وقد أظهرت نتائج الفحص باستخدام البروتوكول الحالي سلبية جميع اللقاحات المختبرة لوجود ملوثات ، وكانت هذه النتائج مرضية باستخدام هذا البروتوكول .وباستخدام تفاعل البلمرة المتسلسل للكشف عن فيروس الرتيكيلواندوثليوزيس كملوث لتلك اللقاحات جاءت جميع النتائج سلبية عدا لقاح واحد مشكوك في تلوثه.فتم حقن هذا اللقاح على البيض المخصب وخلايا الزرع النسيجي الأولية والكتاكيت الخالية من المسببات المرضية لثلاثة تمريرات وبفحص العينات المجمعة من العائل أكدت جميع النتائج إيجابية تلوث اللقاح بفيروس ليكوزيس الدجاج.وقد أظهرت هذه الدراسة أهمية استخدام تفاعل البلمرة المتسلسل المتبوع لتحليل التتابع الجيني المنتجات الذي تم إكثاره للتأكيد على وجود التلوث في لقاحات فيروس جدري الدجاج.