Use Of Polymerase Chain Reaction For Detection Of Contamination In Some Attenuated Avian Vaccines By Infectious Laryngotracheitis Virus

Badawi, A.A.; Magda M. Sayed; Elham A. El-Ebiary and Taha, M.M.
Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo

ABSTRACT

Polymerase chain reaction (PCR) was performed for detection of infectious laryngotracheitis virus (ILTV) in deliberately contaminated vaccines. Using pair of primers, ILT P32 U2 and ILT P32 L2, prepared from P32 gene of ILTV a specific product was amplified from both Serva and Connecticut strains of ILTV but not from gallid herpesvirus 2 indicating the specificity of the reaction. The sensitivity of the reaction was evaluated by testing 10-fold dilutions of ILTV and the minimum detection limit was determined. Serva and Connecticut strains were detected up to a dilution of 10^{-6} and 10^{-4} respectively. The two strains were used to contaminate various types of avian viral vaccines and PCR was conducted to evaluate the efficacy of the reaction to detect ILTV in contaminated vaccines and to determine if the vaccine ingredients have a role on the sensitivity of the reaction. Testing of infectious bursal disease (IBD), avian influenza (AI) and egg drop syndrome (EDS) vaccines revealed that vaccine ingredients did not interfered with reliability and sensitivity of PCR. However, in case of fowl pox vaccine a slight decrease in sensitivity was recorded.

INTRODUCTION

Infectious larygotracheitis (ILT) is a respiratory tract infection of chickens caused by DNA virus that may result in severe production losses due to mortality and/or decreased egg production. In mild enzootic forms of infection the clinical signs include nasal discharge and moist rales followed by coughing and gasping. In severe epizootic forms the characteristic signs are marked dyspnoea and expectoration of blood-stained mucous *(1)*. Infectious laryngotracheitis virus (ILTV) has been identified in most countries and remains a serious disease where susceptible poultry populations occur, especially in large numbers *(2)*. Egg transmission of ILTV contained in the interior or exterior of the egg has not been demonstrated but mechanical transmission can occur by use of contaminated equipment and litter *(3)* and consecutively the virus may introduce to flocks through contaminated vaccines. ILTV is a pathogen normally selected for exclusion from specific pathogen free chicken flocks. Therefore, the requirements described for detection of extraneous virus contamination in avian viral vaccines include monitoring for ILTV *(4)*. Detection of ILTV in possibly contaminated vaccines is currently achieved by either egg or chicken inoculation *(5-7)*. Although these methods are established and routinely used, they are tedious and labor intensive and pose an ethical problems. Furthermore, the practice of vaccine neutralization with specific antiserum, which is an obligatory step in egg inoculation procedures, is considered a great obstacle and initiate significant problems. For these reasons, the *in-vivo* tests should be replaced by *in-vitro* detection systems that may overcome these difficulties. Recently, the polymerase chain reaction (PCR) has been developed which enables detection of ILTV *in-vitro (8-10)*. PCR based technology has been described for the detection of Newcastle disease virus *(11)*, canine parvovirus *(12)*, infectious bronchitis virus *(13)* and avian leucosis virus *(14)* in vaccine preparations. The objective of the present study was the application of PCR for the quality control of avian virus vaccines to test for the presence of contaminating ILTV as an alternative to *in-vivo* tests.

MATERIAL AND METHODS

1. Viruses

Two strains of ILTV, Serva strain contained $10^{8.2}$ EID_{50}/ml and Connecticut strain contained $10^{6.2}$ EID_{50}/ml were used as positive

control in the development of PCR. Gallid herpesvirus-2 (strain Rispens) was used parallel to the ILTV to prove the specificity of PCR. All viruses were obtained as commercially available vaccines.

2. Vaccines

Live attenuated and mineral oil inactivated vaccines were used as target material for detection of ILTV contamination. Fowl pox and infectious bursal disease (IBD) were used as model for live vaccines where avian influenza (AI) and egg drop syndrome (EDS) were used as inactivated vaccines.

3. Sample preparation

Live attenuated vaccines were rehydrated with sterile distilled water (5 ml/1000 dose), while inactivated vaccines were used as it is. ILTV strains were tested in serial ten-fold dilutions ranging from 10^{-1}-10^{-9} and 0.5 ml from each dilution was used to contaminate an equal volume of target vaccines. The procedures of dilution and spiking of vaccines with ILTV were followed *(4)*. Each dilution of ILTV strains was tested twice, before and after mixing with target vaccines.

4. Oligonucleotide primers

Specific primers detecting ILTV gene P32 were designed as described *(4, 15)*. The forward primer ILT P32 U2 (21 mer) is composed of CTA CGT GCT GGG CTC TAA TCC and the reverse primer ILT P32 L2 (21 mer) is composed of AAA CTC TCG GGT GGC TAC TGC. Oligonucleotide primers were synthesized by Bio.Basic.Inc.

5. DNA extraction

DNA from all samples including dilutions of ILTV, gallid herpesvirus 2, spiked and non-spiked vaccines was extracted with the FAVNK001-1 viral nucleic acid extraction kit (Favorgen Biotech USA) according to the manufacturer's protocol.

6. PCR reaction

PCR was conducted as described previously *(4)* as follow: The reaction mixture contained 14µl of sample, 1 µl (0.4 mM) of each deoxynucleoside triphosphate, 1 µl (30 pmol) of primer ILT P32 U2, 1 µl (30 pmol) of primer ILT P32 L2, 5 µl 10x PCR buffer (30 mM KCl; 1.5mM MgCl2; 10 mM Tris-HCl pH 9.0, Bioron, Germany), 0.5 µl Klein Taq polymerase (5 units/µl Bioron, Germany) and a 50 µl total volume was obtained by adding DEPC-treated water. The temperature profile consisted of an initial denaturation step at 95°C for 5 minutes followed by 35 cycles for 30 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C. The PCR product was resolved by electrophoresis in 2% agarose gel and stained with ethidium bromide and examined in an UV transilluminator.

RESULTS

1. Specificity of PCR

PCR amplification of DNA from Serva and Connecticut strains generated a 588 basepair fragment specific for ILTV. Specificity of the reaction was confirmed by testing DNA from gallid herpesvirus 2 and negative control. As shown in Fig. 1, a clear band was detected with ILTV but not with either gallid herpesvirus 2 or negative control.

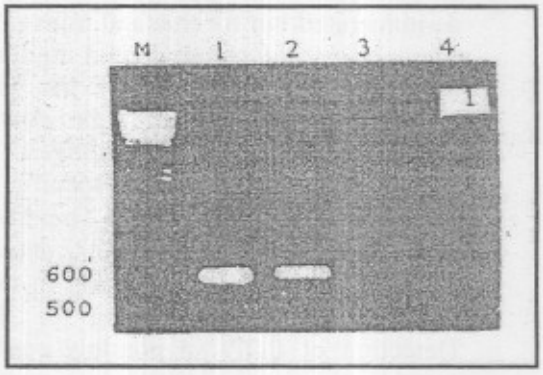

Fig. 1. Specificity of PCR to detect ILTV. Serva strain (lane 1), Connecticut strain (lane 2), gallid herpesvirus 2 (lane 3), negative control (lane 4), 100 bp DNA marker(Roche applied science lane M).

Table 1. Limits of sensitivity of PCR analysis for detection of ILTV in stock virus preparations and in experimentally contaminated vaccines.

Virus strains	Detection limits of ILTV				
	In 10-fold dilutions from 10^{-1} through 10^{-9}	In vaccines experimentally contaminated with ILTV			
		IBDV	FPV	AIV	EDSV
Serva	Up to 10^{-6}	Up to 10^{-6}	Up to 10^{-4}	Up to 10^{-6}	Up to 10^{-6}
Connecticut	Up to 10^{-4}	Up to 10^{-4}	Up to 10^{-3}	Up to 10^{-4}	Up to 10^{-4}

2. Sensitivity of PCR

To determine the sensitivity limits of the reaction, 10-fold dilutions ranging from 10^{-1}-10^{-9} were prepared from Serva and Connecticut strains and PCR was conducted. As shown in Fig. 2, Serva strain could be detected up to a dilution of 10^{-6} while Connecticut strain was detected up to 10^{-4}dilution .

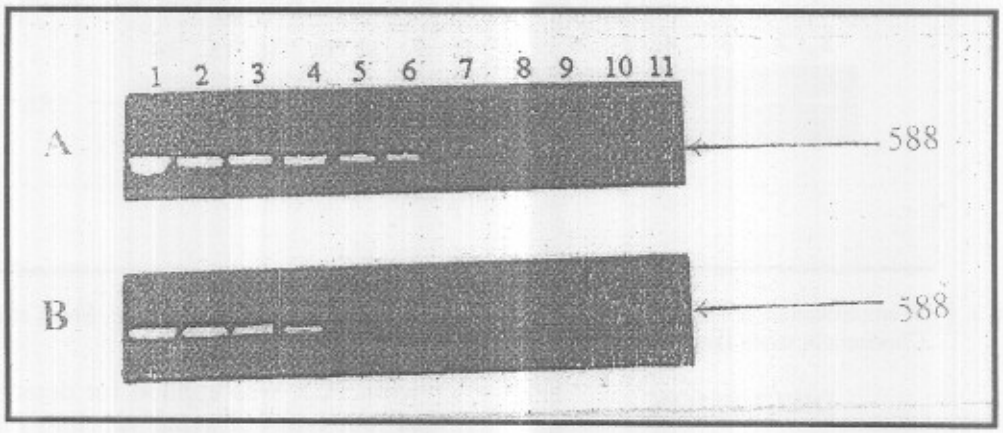

Fig. 2. Sensitivity of PCR to detect the minimal limit of ILTV strains in serial 10-fiold dilutions from 10^{-1}-10^{-9}. A, dilutions of Serva strain (lanes 1-9), B, dilutions of Connecticut strain (lanes 1-9), Negative control (lane 10)

3. Detection of ILTV in contaminated vaccines

Samples of live vaccines (Fowl pox and IBD) as well as mineral oil-inactivated vaccines (AI and EDS) were subjected to routine examination for detection of extraneous virus contamination as described in *(5,6,7)*. All vaccines were found free from extraneous virus contamination. Techniques, host systems and results of routine examination are shown in table 2. After being examined for extraneous virus contamination, each vaccine sample was spiked with 10-fold dilutions of either Serva or Connecticut strains and were tested by PCR to determine whether the vaccine ingredients can interfere with the sensitivity of the reaction or not. In IBD, AI and EDS vaccines, Serva strain could be detected up to a dilution of 10^{-6} in each, while Connecticut strain could be detected up to a dilution of 10^{-4} in each (table 1). The limits of sensitivity are the same that have been obtained by testing of ILTV strains alone, indicating that the ingredients of these three types of vaccines did not interfere with PCR sensitivity. However, in testing of fowl pox vaccines, Serva and Connecticut strains could be detected up to a dilution of 10^{-4} and 10^{-3} respectively (Table 1 and Fig. 3).

Table 2. Detection of extraneous virus contamination in some viral vaccines using different techniques.

Vaccines tested			Results of testing by different techniques		
Vaccine	Type	Batch no.	TC* inoculation	Egg** inoculation	Chicken*** inoculation
IBD	Live	1129068412	-ve	-ve	-ve
Fowl pox	Live	0119099012	-ve	-ve	-ve
AI	Inactivated	02100798	NT •	NT	-ve
EDS	Inactivated	11080187	NT	NT	-ve

*Culture fluid was tested at 7-9 days by ELISA for detection of ALV group specific antigen.
** Allantoic fluid was tested by HA for detection of hemagglutinating agents and embryos were examined for pathological lesions.
***Serum samples were tested by ELISA for detection of antibodies against extraneous agents.
• NT = not tested.

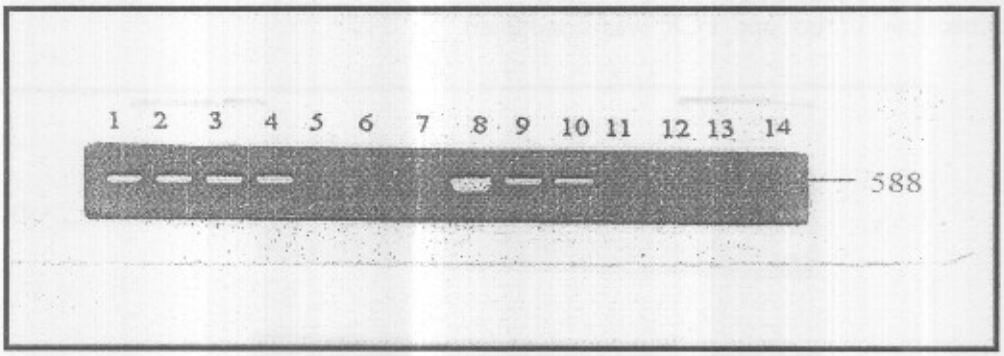

Fig. 3. PCR amplification of 10-fold dilutions of ILTV strains in fowl pox virus vaccine. Serva strain (lanes 1-6), Connecticut strain (lanes 8-13), negative control (lanes 7 and 14)

DISCUSSION

The requirements for detection of extraneous virus contamination in avian viral vaccines are stated in the European Pharmacopoeia, Code of Federal Regulations and the British Pharmacopoeia (***5-7***). Methods described in these texts for detection of ILTV contamination consists of chicken inoculation and testing of serum for ILTV antibodies. Although these methods are routinely used, they are time consuming and laborious. Furthermore, serological tests are hampered by non-specific or cross-reactions ***(16).*** For these reasons the in-vivo methods should be replaced by in-vitro techniques. Polymerase chain reaction has been described as rapid and simple *in-vitro* technique for detection of vaccine contamination with various pathogens including the ILTV ***(8, 14).*** In the present work PCR was applied for detection of ILTV in four types of avian vaccines that have been deliberately contaminated with the virus. Two primers were utilized in the reaction, ILT P32 L2 and ILT P32 U2 which were designed from a part of P32 gene of ILTV. Analysis by agarose electrophoresis indicated that a DNA fragment of 588 bp was amplified when the DNA extracted from ILTV strains was subjected to PCR. DNA samples from gallid herpesvirus 2 (strain Rispens) and from distilled water was not amplified indicating the specificity of the reaction for ILTV but not for other herpesviruses. ***Vogtlin et al. (4)*** utilized these primers in PCR reaction for detection of different isolates of herpesviruses including equine herpesviruses 1 and 4, feline rhinotracheitis, bovine herpesvirus 1, porcine herpesvirus, gallid herpesvirus 2 and ILTV. They reported that the reaction is specific for

ILTV only and added that although the P32 gene of ILTV shows homologies to the gx gene from other herpesviruses, no bands could the detected with PCR using templates of these other viruses. To evaluate the sensitivity of PCR to detect the minimal amount of ILTV, ten-fold dilutions ranged from 10^{-1}-10^{-9} was assayed. Results showed that the reaction is more sensitive for detection of Serva strain than for Connecticut strain. This difference in sensitivity in between the two strains may be due to that the original stock of Serva strain was higher ($10^{8.5}$ EID_{50}/ml) than that of Connecticut strain ($10^{6.2}$ EID_{50}/ml).

These results are in agreement with that obtained in previous investigation which (*4*) recorded a difference in sensitivity of PCR to detect different strains of ILTV the difference in infectivities of the viruses. To determine whether the vaccine ingredients have an effect on the efficacy and sensitivity of the reaction, different types of live and inactivated vaccines were experimentally contaminated with ILTV and PCR was conducted. Screening of IBD, AI, and EDS vaccines revealed that vaccine ingredients have no negative effect on reliability and sensitivity of the reaction. ***Vogtlin et al. (4)*** found that the reliability and sensitivity of PCR did not affected by vaccine ingredient when they used this technique to screen water-based live IBD, and mineral oil-inactivated infectious bronchitis vaccines.

Testing of fowl pox vaccine recorded a slight decrease of sensitivity limit. The real cause of this discrepancy in results is not known exactly but the large molecular size of fowl pox virus or the substrate used in fowl pox vaccine preparation may play a role in this difference in PCR sensitivity.

In conclusion, PCR seems to be a rapid, reliable and simple alternative to current *in-vivo* tests for detection of ILTV in live avian vaccines.

REFERENCES

1. ***Bagust, T.J. and Guy, J.S. (1997):*** Laryngotracheitis. In Diseases of Poultry, 10th Ed. pp. 527-539.
2. ***Biggs, P.M. (1982):*** The World of Poultry Disease. Avian Pathol., 11: 281-300.
3. ***Kingsbury, F.W. and Jungherr, E.L. (1958):*** Indirect transmission of infectious laryngotracheitis in chickens. Avian Dis., 2: 54-63.
4. ***Vogtlin, A.; Bruckner, L. and Ottiger, H. (1999):*** Use of polymerase chain reaction (PCR) for the detection of vaccine contamination by infectious laryngotracheitis virus. Vaccine, 17: 2501-2506.
5. ***European Pharmacopoeia (1997):*** 3rd Ed. Strasbourg, France, European Pharmacopoeia Secretariat.
6. ***Code of Federal Regulations (1996):*** Animals and Animal Products. Part 113, Title 09, USA.
7. ***British Pharmacopoeia, Veterinary (2005):*** Appendix XVIB: A24-A27.
8. ***Abbas, F.; Andreasen, Jr and Jackwood, M.W. (1996):*** Development of a polymerase chain reaction and a non-radioactive DNA probe for infectious laryngotracheitis virus. Avian Dis., 40: 56-62.
9. ***Alexander, H.S. and Nagy, E (1997):*** Polymerase chain reaction to detect infectious laryngotracheitis virus in conjunctival swabs from experimentally infected chickens. Avian Dis., 41: 646-653.
10. ***Scholz, E.; Porter, R.E. and Guo, P. (1994):*** Differential diagnosis of infectious laryngotracheitis from other avian respiratory diseases by simplified PCR procedure. J. Virol. Methods, 50: 313-321.
11. ***Stäuber, N.; Brechtbühl, K.; Bruckner, L. and Hofmann, N.A. (1995):*** Detection of Newcastle disease virus in poultry vaccines using the polymerase chain reaction and direct sequencing of amplified cDNA. Vaccine, 13: 360-364.
12. ***Senda, M.; Parrish, C.R.; Harasawa, R.; Gamoh, K.; Muramatsu, M.; Hirayama, N. And Itoh, O. (1995):*** Detection by

PCR of wild-type canine parvovirus which contaminates dog vaccines. J. Clin. Microbiol., 33: 110-113.

13. ***Falcone, E.; D'Amore, E.; DiTrani, L.; Puzelli, S. and Tollis, M. (1996):*** Detection of avian infectious bronchitis virus in poultry vaccines by polymerase chain reaction. ATLA-Altern. Lab. Anim., 24: 136.
14. ***Häuptli, D.; bruckner, L. and Ottiger, H.P. (1997):*** Use of reserve transcriptase polymerase chain reaction for detection of vaccine contamination of avian leucosis virus. J. Virol. Methods, 66: 71-81.
15. ***Kongsuwan, K.; Johnson, M.A.; Prideanx, C.T. and Sheppard, M. (1993):*** Identification of an infectious laryngotracheitis virus gene encoding an immunogenic protein with a predicted M(r) of 32 kilodaltons. Virus Res., 29: 125-140.
16. ***Xie, Z.; Khan, M.I.; Girshick, T. and Xie, Z. (2005):*** Reverse transcriptase polymerase chain reaction to detect avian encephalomyelitis virus. Avian Dis., 49: 227-230.

الملخص العربى

استخدام اختبار تفاعل البلمرة المتسلسل لكشف تلوث بعض لقاحات الدواجن بفيروس التهاب الحنجرة والقصبة الهوائية المعدى

*** احمد عبد اللطيف بدوى** *** ماجدة محمد سيد**

*** إلهام عطا الإبيارى** *** محمد محمود طه**

المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية – العباسية – القاهرة

تم استخدام تفاعل البلمرة المتسلسل للكشف عن فيروس التهاب الحنجرة والقصبة الهوائية المعدى فى عينات لقاح تم تلويثها معملياً بالفيروس. وقد تم استخدام عترتين للفيروس هما عترة Serva وعترة Connecticut وكذلك تم استخدام بادئ متخصص للفيروس تم تحضيره من بروتين الجين 32 P32 gene. وقد أثبتت الاختبار حساسيته وخصوصية فقط لفيروس التهاب الحنجرة والقصبة الهوائية المعدى وذلك بعدم الحصول على نتيجة إيجابية عند استخدام هذا البادئ مع فيروس gallid herpesvirus-2. كما اثبت الاختبار ان المكونات الداخلة فى تركيب لقاحات الجمبورو والأنفلونزا وتدنى البيض لم تؤثر بالسلب على حساسية وفاعلية الاختبار إلا ان لقاح جدرى الطيور سجل نقص بسيط فى حساسية الاختبار.