Application of Reverse Transcriptase Polymerase Chain Reaction to Detect Avian Encephalomyelitis Virus in Some Poultry Vaccines

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ABSTRACT

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used for detection of Avian encephalomyelitis virus (AEV) contamination in some live poultry vaccines. The AEV RT-PCR was found to be sensitive to detect as little as 100 picogram (pg) of AEV RNA in deliberately contaminated poultry vaccines. Using pair of AEV-specific primers agarose gel electrophoresis analysis indicated that a DNA fragment of 619 base pair specific for AEV was amplified from Calnek 1143 strain of the virus. RNA from infectious bronchitis virus, infectious bursal disease virus and Newcastle disease virus didn`t amplified the specific DNA fragments under the same conditions. The presence of these three viruses in the reaction didn`t interfere with the efficacy of the technique to detect AEV as a contaminant.

INTRODUCTION

Avian encephalomyelitis (AE) is a viral infection occurs in chickens, turkeys, pheasant and quail . Infection of young chickens causes clinical signs of ataxia, in-coordination, paralysis and tremors of head and neck. In adults laying birds, AE infection causes a slight reduction in egg production. Up till now the detection of Avian encephalomyelitis virus (AEV) as an extraneous agent in avian vaccine required either the embryo inoculation and observation of hatched chicks for AEV-specific symptoms or the use of chicken inoculation assay and testing of serum for AEV-specific antibodies *(1-3)* Although these methods are established and routinely used they are time consuming and labor intensive. Furthermore, serological test are often hampered by non specific reaction and therefore should be replaced by *in vitro* detection system *(10)*. In recent years, the polymerase chain reaction (PCR) method has been applied as a rapid diagnostic tool for the detection of many viral and bacterial pathogens *(5-9)*. A specific reverse transcriptase (RT) PCR was developed for detection of AEV by inoculation of suspected samples into chicken embryos and testing brain tissues of the inoculated embryos for AEV-specific RNA *(10)*. Although the described technique is specific and sensitive, it still confined to use for virus diagnosis in field samples and not yet used for detection of AEV as a contaminant in viral poultry vaccine.

The aim of present study was to evaluate the application of RT-PCR method to test some poultry vaccines for the presence of contaminating AEV as an alternative to the current test in use.

MATERIAL AND METHODS

1-AE virus

Calnek 1143 strain of AEV was served as positive control in the development of the RT-PCR. The virus was obtained commercially as lyophilized vaccine (IZO S.P.A. Italy) and it was rehydrated in sterile distilled water, so that 1ml contained $10^{5.5}$ EID50. The stock virus was aliquated in 1ml volume and stored at -20C° until used.

2-Vaccines

Twenty eight batches of RNA-viral vaccines were used as target samples for the detection of AEV as possible contaminant. The type and number of tested batches of vaccines are shown in Table 1.

Table 1. List of types and numbers of vaccines used for detection of AEV as possible contaminant.

Type of vaccine	No. of tested batches	Code number
IB	3	0123018712, 0123038212, 0187048412
ND	6	0112078512, 0112058612, 0123048512, 0123038512, 0124048012, 0145008012,
IBD	19	0123058312, 0123058912, 0123068012, 0123068712, 0153088912, 1114008212, 1114008012, 1114008712, 1114018912, 0124038712, 0124038912, 0144058812, 0144068012, 0144068112, 0144098212, 0144098312, 0144098712, 0145018712, 0155038812

IB: ND: Newcastle IBD

3-AEV primers

Pair of primers that specifically amplified AEV were designated as MK AE1(CTT ATG CTG GCC CTG ATC GT) and MK AE2 (TCC CAA ATC CAC AAA CCT AGCC) and selected on the bases of the published sequence data of AEV *(4)*.

4-RNA Extraction

For RNA extraction, all samples including target vaccines and AEV stocks were processed with Axy prep body fluid viral DNA/RNA mini prep kit (Axygen Biosciences, Central Avenue, Union City. USA) according to the manufacturer's protocol.

5-RT-PCR

Two-steps RT-PCR was conducted using Bioron GmbH PCR kit. The first step is a reverse transcription which was followed by a second PCR reaction. In the reverse transcription step the reaction mixture contained 2µl of 10X reverse transcription buffer (100mM Tris-HCL [PH 8.9], 900 mM KCL),4µl of 25 mM MnCL,2µl of 10mM of each deoxynucleoside triphosphate (dNtps), 1µl reverse primer MK AE2, 1µl (5 units) of Tth DNA polymerase, and 100 ng of RNA. A total volume of 20 µl reactions was obtained by adding sterile H_2O. The reverse transcription was carried out by incubation of the reaction tube at 62°C for 30 min. For the PCR reaction, 10µl of 10 X PCR buffer (100 mM tris HCL [PH 8.9], 1M KCL, 500 µg/ml B.S.A, 0.5% tween 20 and 15 mM $MgCl_2$), 10 µl of a 7.5 mM EGTA solution and 750 nM of forward primer MK AE1, were added to the above RT reaction tube and a 100µl total volume was obtained by adding sterile distilled water. The reaction mixture was denatured at 94°C for 2 min. then the PCR was run for 30 cycles at 94°C for 30 sec., 60°C for 30 sec., and 65°C for 1 min. for denaturation, annealing and elongation, respectively. Then at 62°C for 10 min. for one cycle.

6-Sensitivity of AEV RT-PCR

Ten fold dilutions starting from 100ng to 10 pg of AEV RNA were subjected to AEV RT-PCR to check the sensitivity of the technique.

RESULTS

Analysis of 28 batches of various RNA viral vaccines indicated that all of them are free from AEV contamination. No amplification for any RNA extracted from these vaccines was seen, while cDNA fragment of approximately 619 base pair specific for AEV was amplified from the positive control under the same conditions as shown in Fig (1).

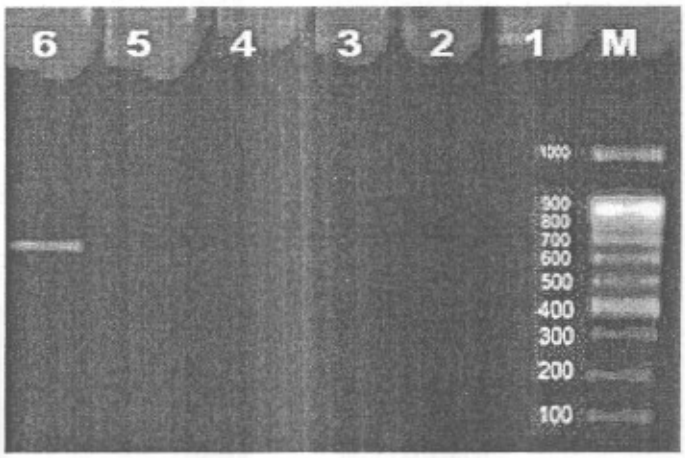

Fig.1. Agarose gel electrophoresis of RT PCR of different vaccine batches, Only AEV positive control was amplified (Lane 6). M: marker

The specificity of AEV RT-PCR was evaluated in another set of the experiment when stocks of AEV was added to these negative vaccines and all samples were retested under the same conditions as before. As shown in Fig. 2, RNA from IBV, IBDV and NDV were not amplified while cDNA fragment specific for AEV was obtained from the positive controls and from the experimentally-contaminated vaccines but not from uncontaminated samples No PCR, amplification reaction was seen in all negative controls.

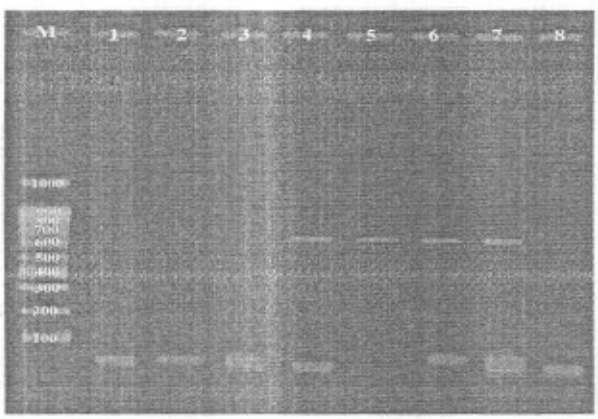

Fig. 2. Specificity of RT-PCR for detection of AEV in various vaccine samples. Lane M DNA marker, Lane (1)IBV, Lane (2) IBDV, Lane (3) NDV, Lane (4) IBV+AEV, Lane (5) IBDV+AEV, Lane (6) NDV+AEV, Lane (7) AEV, Lane (8) negative control.

The sensitivity of AEV RT-PCR to detect the minimum amount of AEV-RNA was evaluated. Results in Fig.3 showed that a minimum amount of 100 pg of AEV-RNA could be detected as determined by electrophoresis.

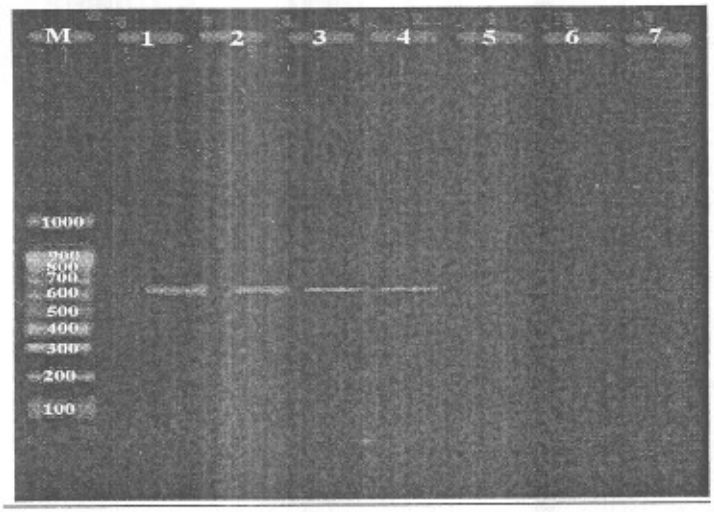

Fig.3. Limits of sensitivity of AEV RT-PCR to detect the minimum amount of AEV-RNA. Lane M DNA marker, Lane (1) 100ng, Lane (2) 10 ng, Lane (3)1ng, Lane (4) 100pg, Lane (5)10pg, Lane (6) negative control.

DISCUSSION

An alternative *in vitro* technique is necessary to overcome the disadvantages of *in vivo* assay used for detection of AEV as vaccine contaminant. The *in vivo* assays include the chick and/or embryo inoculation ***(1-3)***. The RT-PCR for detection of AEV has been developed ***(10)***, but still limited to use for diagnosis of the virus in field samples. In a trial to maximize the benefit of the technique we used one pair of AEV primers to detect the virus as a contaminant in some poultry vaccines by using the AEV RT-PCR. Results indicated that the technique is specific and sensitive. The specificity was proved by detection of AEV-DNA fragment only when AEV was included in the reaction. No amplification occurred for any of IBV, IBDV or NDV RNA under the same conditions. Furthermore, when IB, IBD and ND vaccines were deliberately contaminated with AEV a specific fragment for AEV was detected regardless the type of vaccine, indicating that the vaccine ingredients didn't interfere with the results of AEV RT-PCR. This is an important aspect because the currently used methods (*in vivo* procedures) require the vaccine to be effectively neutralized with specific antiserum before testing for extraneous virus contamination. Because the vaccine neutralization is considered the most critical step in the techniques, so, the RT-PCR is the test of choice to overcome this problem. The sensitivity of the test was checked and a minimum amount (100 pg) of AEV-RNA could be detected. These results are in accordance with reports described for the use of AEV RT-PCR to detect the virus in field samples in which a minimum amount of 10 pg of viral RNA was detected ***(10)***. The present communication indicates that the RT-PCR is equally applicable to detect AEV as contaminant in live poultry vaccines.

In conclusion; a small amount of RNA could be amplified directly from the vaccine samples without special treatment and so this *in vitro* technique may replace the current tests in use.

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الملخص العربى

تطبيق استخدام تفاعل البلمرة المتسلسل العكسى للكشف عن فيروس الارتعاش الوبائى فى بعض لقاحات الدواجن

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تم استخدام اختبار البلمرة المتسلسل العكسى للكشف عن فيروس الارتعاش الوبائى كملوث فيروسى فى بعض اللقاحات الحية للدواجن باستخدام بادئ متخصص بفيروس الارتعاش الوبائى؛ وقد اثبت الاختبار دقة وحساسية كبيرة فى الكشف والتعرف على الفيروس فى اللقاحات التى تم تلويثها معمليا بفيروس الارتعاش الوبائى اما اللقاحات الغيرملوثة فاعطت نتيجة سلبية. ولتقييم حساسية الاختبار قد تم اجراؤه على تخفيفات متناقصة من الحمض النووى للفيروس وكان الحد الادنى الذى تم اكتشافه هو ١٠٠ بيكو جرام فى العينة . اما بالنسبة للتخصص فقد تأكدت خصوصية الاختبار فى الكشف عن فيروس الارتعاش الوبائى فقط ولم يتم الحصول على نتيجة ايجابية عند استخدام نفس البادئ فى التفاعل مع اى من فيروس النيوكاسل والالتهاب الشعبى او الجمبورو؛ كما ان وجود هذه الفيروسات فى التفاعل لم يؤثر على كفاءة الاختبار فى التعرف على فيروس الارتعاش الوبائى فى اللقاحات المختلفة.