*Bulgarian Journal of Veterinary Medicine*, 2020, **23**, No 3, 304–309
ISSN 1311-1477; DOI: 10.15547/bjvm.2230

*Original article*

# USE OF DEGENERATE OLIGONUCLEOTIDE PRIMED POLYMERASE CHAIN REACTION FOR DETECTION OF CHICKEN ANAEMIA VIRUS CONTAMINATION IN AVIAN VIRAL VACCINES

M. JAMSHIDIAN-MOJAVER[1], S-E. TABATABAEIZADEH[1], M. NAEEMIPOUR[2], H. R. FARZIN[1] & M. R. BASSAMI[3]

[1]Mashhad Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran; [2]Department of Biochemistry and Nutrition, Cellular and Molecular Research Center, Sabzevar University of Medical Sciences, Sabzevar, Iran; [3]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

**Summary**

Jamshidian-Mojaver, M., S-E. Tabatabaeizadeh, M. Naeemipour, H. R. Farzin & M. R. Bassami, 2020. Use of degenerate oligonucleotide primed polymerase chain reaction for detection of chicken anaemia virus contamination in avian viral vaccines. *Bulg. J. Vet. Med.,* **23**, No 3, 304–309.

For quality control of biologicals of veterinary use, the absence of extraneous agents needs to be certified. One of the requirements for quality control of avian viral vaccines is to demonstrate freedom from extraneous and adventitious pathogenic agents, like chicken anaemia virus (CAV). In this study, a degenerate oligonucleotide primed PCR (DOP-PCR) for the detection of CAV was developed. Degenerate oligonucleotide primers were selected based on sequences corresponding to conserved regions of VP1 gene. After spiking of CAV genomic DNA to an infectious laryngotracheitis virus (ILTV) vaccine, detection limit for the test was $3.056\times10^{-9}$ ng/µl. To evaluate the performance of the test, 11 avian viral vaccines including infectious bronchitis virus (IBV), newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and ILTV vaccines from 5 manufacturers were screened for CAV and no contamination was detected. The test described here may provide a rapid, sensitive and specific method for contamination detection of avian viral vaccines with CAV, and may be applied for quality control of live and killed commercial vaccines.

**Key words**: avian viral vaccines, chicken anaemia virus (CAV), quality control

## INTRODUCTION

CAV is a non-enveloped virus, and its genomic features are more closely aligned with ssDNA viruses in the family *Anellviridae* and, thus, the genus *Gyrovirus* was reassigned to this family (Rosario *et al.,* 2017). Chicken infectious anaemia (CIA) caused by CAV is a disease of young chickens (De Herdt *et al.,* 2001; Schat *et*

*al.,* 2011). The causative agent was first isolated in 1978 (Taniguchi *et al.,* 1982). CIA is characterised by anaemia, severe immunosuppression and marked atrophy of bone marrow, thymus, and bursa of Fabricius (Taniguchi *et al.,* 1982; Cardona *et al.,* 2000a; Schat *et al.,* 2011). The immunosuppression is responsible for increased mortality, reduced performance and decreased resistance to viral and bacterial diseases during the breeding period (Hussein *et al.,* 2003).

Viral contamination of human, mammalian and avian vaccines have been reported by several researchers. Due to the vast usage of embryonated chicken eggs and primary cell cultures for avian vaccine production, there is a high risk of vaccine contamination. Frequent reports of vaccine contamination have led the FDA to name 2003 as the year of contamination of specific pathogen free eggs (Yuasa & Yoshida, 1983; Chandratilleke *et al.*, 1991; Calnek *et al.,* 2000; Hagood *et al.,* 2000; Miles *et al.,* 2001).

Viral safety of biologicals includes certification of freedom from extraneous agents. According to European Pharmacopoeia, evaluating at least 16 pathogens including CAV is essential for quality control of vaccines (Anonymous, 1998; 1999). Methods for detection of extraneous agents include serologic tests, viral isolation in inoculated birds, embryonated chicken eggs or cell cultures. The conventional assays are laborious, time-consuming and need animals to be performed. To replace, reduce or refine the use of animals in quality control of biologicals including vaccines (the 3Rs approach), an alternative testing scheme using PCR has been proposed for many contaminants (Bruckner & Kihm, 1986). The European Pharmacopoeia, United States Pharmacopoeia and British Pharmacopoeia allow the use of alternative methods, including PCR, with competent authority´s approval and fully validated.

Degenerate oligonucleotide-primed PCR (DOP-PCR) employs oligonucleotides of partially degenerate sequence. Furthermore, efficient amplification is achieved from the species genomes using the same primers. DOP-PCR appears to have advantages over interspersed repetitive sequence PCR (IRS-PCR), which relies on the appropriate positioning of the genome. DOP-PCR therefore represents a rapid, efficient, and species-independent technique for general DNA amplification.

The test described in this paper, may provide a rapid, sensitive and specific method for contamination detection of avian viral vaccines with CAV, and may be applied for quality control of commercial vaccines.

## MATERIALS AND METHODS

*Primer design:* Fifty CAV sequences retrieved from GenBank and aligned using ClustalW. These sequences were divided into two groups based on similarities, and two sequences (Accession numbers AY739211.1 & D31965.1) with the most similarities to each of the two groups were selected for primer design. Two degenerate primers homologous to the conserved sequences within VP1 selected that amplify a 676 bp segment. Primer specificity was checked using Primer-BLAST. The sequence of forward and reverse primers were 5'-GACTGT(A,G)AGATGG(A,C) AAGACGAGCTC-3' and 5'-G(A,G)CTG AAGGATCCCTCATTC-3', respectively.

*DNA extraction and amplification:* DNA was extracted from chicken liver and vaccine samples including CAV vaccine, ILTV vaccine, IBV vaccine, NDV vaccine, IBDV vaccine using the standard

phenol-chloroform method (Sigma-Aldrich, Germany).

*Optimisation of PCR conditions:* To find the best conditions for PCR reaction, 16 reactions with different $MgCl_2$ concentrations and buffer pH values were investigated (PCR Optimization Kit, Roche, Germany). The next step, 3 additional additives (DMSO, Glycerol, Gelatin) were tested to increase yield and specificity. DMSO reduces nonspecific priming, while glycerol and gelatin increase the yield by stabilising Taq DNA polymerase during PCR (PCR Optimization Kit, Roche, Germany).

*Polymerase chain reaction:* The PCR assay was carried out in a final volume of 25 µL mixture consisting of PCR buffer (10 mM Tris-HCl, pH 8.3), 1.0 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 10 pM of each primer, 1.25 U of Taq DNA polymerase and 2 µL of template. The amplification was performed under the following conditions in a thermal cycler (Bio-Rad Co.,USA): a denaturation step of 95 $^oC$ for 5 min followed by 35 cycles of 94 $^oC$ for 30s, 58 $^oC$ for 30 s, 72 $^oC$ for 90 s, with a final extension at 72 $^oC$ for 10 min. The PCR product was then analysed by electrophoresis in 1.5% agarose gel and visualised under ultraviolet light after staining with ethidium bromide. Intensity analysis of PCR bands performed using the Phoretix 1D Pro gel analysis software (TotalLab, England) based on the brithness of each band. The PCR product was sequenced (Macrogen, Korea) to confirm the amplification of VP1 sequence.

12srRNA gene primers were used as an internal control for PCR reaction using the extracted genomic DNA of chicken liver and each vaccine sample as template.

*Evaluation of the detection limit of PCR:* VP1 gene PCR product was extracted from the gel and quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration was 15.28 ng/µL. The detection limit was evaluated by preparing 10-fold serial dilutions of the PCR product and spiking 2 µL of each dilutions to ILTV vaccine (Razi Vaccine and Serum Research Institute, IRI) sample. This was done to mimic a situation of vaccine contamination.

To evaluate a number of vaccines currently used in poultry industry in Iran, three samples of each 11 avian viral vaccines including ILTV (Razi Vaccine and Serum Research Institute, IRI; Ceva), IBV (Razi Vaccine and Serum Research Institute, IRI), NDV (Razi Vaccine and Serum Research Institute, IRI; Lohmann Animal Health; HIPRA) and IBDV (Fort Dodge) vaccines from 5 manufacturers were screened for CAV contaminantion using the developed DOP-PCR.

## RESULTS

Before the optimisation steps, PCR was performed to confirm the designed degenerate primer pair in the amplification of the 676 bp segment. Fig. 1A shows the 676 bp PCR product of CAV primers after optimization steps. A PCR reaction done using a primer pair for 12srRNA gene as an internal control. It also shows that the designed degenerate primers specifically amplified the target sequence.

At the first step of PCR optimisation, different magnesium and pH concentrations were examined. Based on the PCR reaction performed with buffer containing 1.0 mM of $MgCl_2$ and pH value of 9.2 gave the strongest PCR product band at an annealing temperature of 58 $^oC$. At the second step of PCR optimisation, 3 additional additives (DMSO 5%, gelatin 0.05%

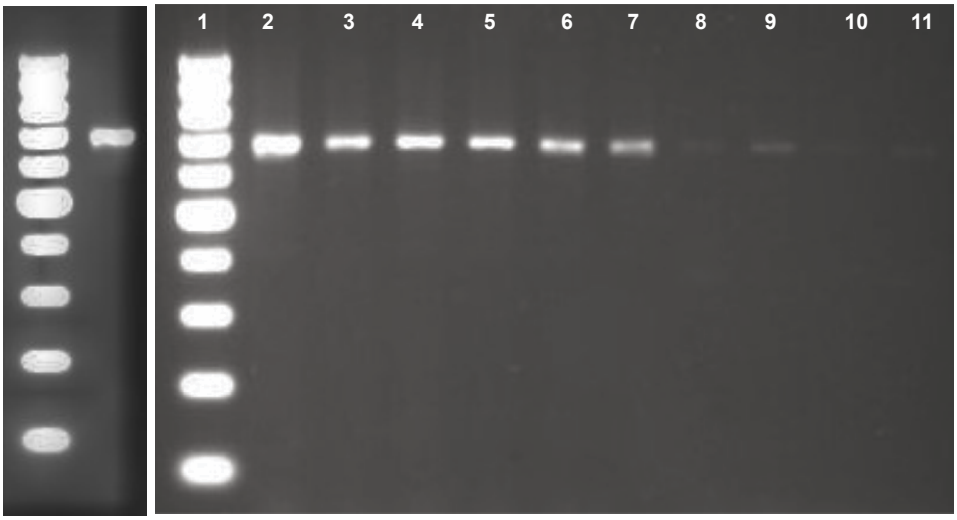


**Fig. 1.** A) PCR product of CAV primers after optimisation. B) Detection limit of the test; the concentration corresponding to lane 2 is 3.056 ng/µL. The detection limit was evaluated by preparing 10-fold serial dilutions of the PCR product and spiking 2 µL of each dilutions to sample. Developed test could detect CAV DNA at $3.056\times10^{-9}$ ng/µL (Lane 11). Lane 1 is the 100 bp DNA ladder.

and glycerol 10%) were added to PCR with 1.0 mM of $MgCl_2$ and pH value of 9.2. The strongest PCR band was gained with glycerol.

Detection limit of the test was determined to be $3.056\times10^{-9}$ ng/µL of CAV DNA (Fig. 1B).

A total of 11 vaccines including ILTV, IBV, NDV and IBDV vaccines from 5 manufacturers were surveyed for CAV contamination. Despite the confirmation of the stages of work using the 12S rRNA internal control, all tested vaccines were found free of contamination.

## DISCUSSION

During the production process and before market release, freedom of vaccines from extraneous pathogenic agents should be secured to prevent infection of vaccinated birds. Specifc pathogen-free (SPF) eggs have been used for avian vaccine production. CAV infection of SPF flock and embryonated eggs have been reported in several studies (Cardona *et al.,* 2000a,b; Yilmaz *et al.,* 2001). Accordingly, vaccines produced using these eggs may be contaminated with the virus (Li *et al.,* 2017). The contamination of vaccines with CAV can lead to anaemia and mortality in vaccinated birds and, as a result of the suppression of the immune system, may reduce the effectiveness of next vaccinations (Toro *et al.,* 1997). Considering the importance of identifying CAV contamination in avian vaccines, PCR has been used as a fast, sensitive and inexpensive method for contamination detection (Amer *et al.,* 2011; Marin *et al.,* 2013; Varela *et al.,* 2014; Li *et al.,* 2017). In a study by Li *et al.* (2017) investigating the source of CAV infection in chickens, the results of the PCR test for

CAV were positive for a fowl pox vaccine and a Newcastle disease vaccine. It was also observed that the use of these contaminated vaccines can lead to CAV infection in the vaccinated chickens.

In another study, carried out on thirty five vaccines from eight manufacturers, CAV contamination was detected in seven vaccines by PCR (Varela *et al.,* 2014).

In the present study, we developed a DOP-PCR to ensure that all variants of CAV will be detected. The test was optimised using different $MgCl_2$ concentration, pH values and additional additives. PCR optimisation enhanced the performance of the PCR assay. Employing CAV genome, test showed an acceptable PCR sensitivity for contamination detection ($3.056\times10^{-9}$ ng/µL of CAV DNA). Accordingly, our test has a lower limit of detection compared to previous studies, so the test sensitivity is improved. Also, the use of denegerate primers in this study enhances the detection sensitivity of the test.

The test descibed here may provide a simple, rapid, sensitive and specific method for the detection of CAV in poultry vaccines, and may be applied for quality control of live and killed avian vaccines. However, this test is not a validated assay and has to be employed for a vast number of vaccine samples, while it is compared with other conventional methods. Screening of a large number of vaccines at a national level would provide a good opportunity for evaluation of the assay developed and optimised in this project.

## REFERENCES

Amer, H. M., H. M. Elzahed, E. A. Elabiare, A. A. Badawy & A. A. Yousef, 2011. An optimized polymerase chain reaction assay to identify avian virus vaccine contamination with chicken anemia virus. *Journal of Veterinary Diagnostic Investigation*, **23**, 34–40.

Anonymous, 1998. ASEAN Standards For Animal Vaccines, Livestock publication series No. 2A https://report.nat.gov.tw/ReportFront/PageSystem/reportFileDownload/C10603937/007 (19 February 2019 date last accessed).

Anonymous, 1999. Council of Europe (1999). 2.6.4. Avian live virus vaccines: Tests for extraneous agents in batches of finished product. *Pharmeuropa,* **11**, 191–193

Bruckner, L., & U. Kihm, 1986. Alternatives for potency testing of veterinary vaccines: Rabies vaccine as a model. *Developments in biological standardization*, **64**, 93-97.

Calnek, B. W., B. Lucio-Martinez, C. Cardona, R. W. Harris, K. A. Schat & C. Buscaglia, 2000. Comparative susceptibility of Marek's disease cell lines to chicken infectious anemia virus. *Avian Diseases*, **44**, 114–124.

Cardona, C., B. Lucio, P. O'Connell, J. Jagne & K. A. Schat, 2000a. Humoral immune responses to chicken infectious anemia virus in three strains of chickens in a closed flock. *Avian Diseases*, **44**, 661–667.

Cardona, C., W. B. Oswald & K. A. Schat, 2000b. Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. *Journal of General Virology*, **81**, 2067–2075.

Chandratilleke, D., P. O'Connell & K. A. Schat, 1991. Characterization of proteins of chicken infectious anemia virus with monoclonal antibodies. *Avian Diseases*, **35**, 854–862.

De Herdt, P., G. Van den Bosch, R. Ducatelle, E. Uyttebroek & C. Schrier, 2001. Epidemiology and significance of chicken infectious anemia virus infections in broilers and broiler parents under nonvaccinated European circumstances. *Avian Diseases*, **45**, 706–708.

Hagood, L. T., T. F. Kelly, J. C. Wright & F. J. Hoerr, 2000. Evaluation of chicken infec-

tious anemia virus and associated risk factors with disease and production losses in broilers. *Avian Diseases*, **44**, 803–808.

Hussein, H. A., M. M. Youssef, A. Osman, E. A. El-Ebiary & M. A. Shalaby. 2003. Immunopathogenesis of attenuated strain of chicken infectious anemia virus in one-day-old specific-pathogen-free chicks. *Egyptian Journal of Immunology*, **10**, 89–102.

Li, Y., Y. Hu, S. Cui, J. Fu, Y. Wang, Z. Cui, L. Fang, S. Chang & P. Zhao. 2017. Molecular characterization of chicken infectious anemia virus from contaminated live-virus vaccines, *Poultry Science*, **96**, 1045–1051.

Marin, S. Y., P. R. Barrios, R. L. Rios, M. Resende, J. S. Resende, B. M. Santos & N. R. Martinsa, 2013. Molecular characterization of contaminating infectious anemia virus of chickens in live commercial vaccines produced in the 1990s. *Avian Diseases*, **57**, 15–21.

Miles, A. M., S. M. Reddy & R. W. Morgan, 2001. Coinfection of specific-pathogen-free chickens with Marek's disease virus (MDV) and chicken infectious anemia virus: Effect of MDV pathotype. *Avian Diseases*, **45**, 9–18.

Rosario, K., M. Breitbart, B. Harrach, J. Segales, E. Delwart, P. Biagini, and A. Varsani. 2017. Revisiting the taxonomy of the family *Circoviridae*: Establishment of the genus *Cyclovirus* and removal of the genus *Gyrovirus*. *Archives of Virology*, **162**, 1447–1463.

Schat, K. A., N. R. Martins, P. H. O'Connell & M. S. Piepenbrink, 2011. Immune complex vaccines for chicken infectious anemia virus. *Avian Diseases*, **55**, 90–96.

Taniguchi, T., N. Yuasa, M. Maeda, and T. Horiuchi. 1982. Hematopathological changes in dead and moribund chicks induced by chicken anemia agent. *National Institute of Animal Health Quarterly*, **22**, 61–69.

Toro, H., A. M. Ramirez & J. Larenas, 1997. Pathogenicity of chicken anaemia virus (isolate 10343) for young and older chickens. *Avian Pathology*, **26**, 485–499.

Varela, A. P., H. F. Dos Santos, S. P. Cibulski, C. M. Scheffer, C. Schmidt, F. E. Sales Lima, A. D. Silva, P. A. Esteves, A. C. Franco & P. M. Roehe, 2014. Chicken anemia virus and avian gyrovirus 2 as contaminants in poultry vaccines. *Biologicals*, **42**, 346–350.

Yilmaz, H., N. Turan, N. Y. Ozgur, C. R. Helps & O. Akay. 2001. Detection of chicken anemia virus DNA in the thymus of naturally infected chicks in turkey. *Avian Diseases*, **45**, 529–533.

Yuasa, N., & I. Yoshida, 1983. Experimental egg transmission of chicken anemia agent. *National Institute of Animal Health Quarterly*, **23**, 99–100.

Paper received 05.11.2018; accepted for publication 18.01.2019

**Correspondence**:

Majid Jamshidian-Mojaver
Mashhad Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran
e-mail: mjmojaver@yahoo.com