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Assessment of polymerase chain reaction sensitivity for the detection of chicken anaemia virus using different primers for three genes

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ABSTRACT

Chicken Anaemia Virus (CAV) genes cloned plasmid DNA templates (pCR4-CAV-VP1 and pCR4-CAV-VP2&3) were used in this study to develop a sensitive polymerase chain reaction (PCR) for CAV gene detection. A total of nine sets of primers, which include one set of published primers and two sets of designed primers for each gene of CAV, viz. VP1, VP2 and VP3, were used to assess the sensitivity of PCR. The PCR cycle conditions were standardized with the designed primers to get a single, specific sized amplicon for each gene separately. PCR sensitivity assessment was done by making serial 10 fold dilutions of the cloned CAV plasmid templates and subjected to PCR with each set of primers for each gene of CAV. The highest dilution of the CAV plasmid DNA showing a visible PCR amplicon was taken as the detection limit. The results showed that the designed primer VP1.2 was found to be more sensitive for the VP1 gene and the concentration of the plasmid DNA was 0.05 fg/μL or 8.6×10^3 molecules/mL and VP 2.2 was found to be more sensitive for the VP2 gene and the concentration of the plasmid was 5 ag/μL or 9.9×10^2 molecules/mL. In the case of VP3, the published primer VP 3.1 was found to be more sensitive for the VP3 gene and the concentration of the plasmid was 5×10^{-4} ag/μL or 10.5×10^{-2} molecules/mL. The findings of this study may be very useful for diagnostic, sequencing, cloning and expression purposes.

Key words: chicken anaemia virus, polymerase chain reaction sensitivity, primers, amplicon

Introduction

In the last two decades, there has been much discussion on the significance of chicken anaemia caused by the Chicken Anaemia Virus (CAV). CAV is an economically important avian pathogen with worldwide distribution and it causes infectious anaemia and immuno-suppression in young susceptible chicks (BULOW and SCHAT, 1997). Anaemia and immunodeficiency in young chicks are caused by the destruction of erythrocyte

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precursors and thymocytes via the induction of apoptosis. In older chickens, its effects are subclinical, but it does cause severe economic problems. The major economic losses caused by this virus are thus associated with severe immuno-suppression and increased mortality due to secondary infections (ROSENBERGER and CLOUD, 1989).

As the virus is resistant to heat and organic solvents, the virus is difficult to eliminate from a poultry farm once the premises become contaminated. Mechanical transmission of CAV infection from one farm to another through people, equipment and vehicles, facilitates the faster spread of infection and thereby necessitates the need for early detection of infection by the most sensitive detection system. High levels of mortality associated with CAV infection are common due to secondary bacterial infection or other viral infections.

The CAV genome codes for three viral proteins. VP1 is a major capsid protein. VP2 is a non-structural scaffolding protein. Both these proteins are immunogenic proteins inducing neutralizing antibodies and could be exploited for diagnostic purposes. VP3 is a small protein - apoptin, which has an apoptosis inducing property. Detecting any of these genes will confirm CAV infection in a flock. Diagnosis of CAV infection can be made by detecting infectious virus, viral antigen, viral DNA, or virus-specific antibodies (CHETTLE et al., 1989). For routine laboratory diagnosis or for testing vaccines for the presence of extraneous CAV, PCR should be as sensitive as virus isolation; ideally, it should detect a single copy of CAV in a large amount of background DNA (NOTEBORN and KOCH, 1995). Sensitivity of the PCR assay seems to vary depending on the reaction conditions used by each research group (NOTEBORN et al., 1992; THAM and STANISLAWEK, 1992; TODD et al., 1992; SOINE et al., 1993).

PCR has the major advantage of providing a faster and sensitive detection of more fastidious viral pathogens that might require several days and consecutive passages in cell culture for virus isolation and allowing the fast *in vitro* diagnosis of viruses that may not be readily isolated by *in vitro* cell culture. The increasing application of PCR technology in molecular biological research should ensure that its use in diagnosis becomes more widespread. The high sensitivity that is possible with this DNA amplification method may be useful for detecting vertical transmission of CAV through eggs or possible virus latency (TODD et al., 1991).

On the grounds of purity, cloned virus specific DNA is preferable to use. In the absence of samples available for virus isolation, CAV from past cases of interest can be regenerated by PCR and molecular cloning for subsequent biological characterization. At present, different primers for each gene of CAV are in use for diagnostic purposes, for cloning and expression. Hence this study was carried out to find a suitable primer for sensitive PCR detection of CAV genes.

Materials and methods

Source of CAV DNA. Full length CAV genes cloned into the commercially available plasmid vector (pCR®4 - TOPO®) viz. pCR4 - CAV-VP1 and pCR4 - CAV-VP2&3, available from the Department of Animal Biotechnology, Madras Veterinary College, Chennai, India, were used in this study. The details of the published primers (IWATA et al., 1998) used in this study are given in Table 1.

CAV plasmid DNA isolation and quantification. Recombinant CAV plasmid DNA for each gene was extracted from the positive recombinant colonies as per the kit protocol (Plasmid isolation kit, Cat. No. K2100-10, Invitrogen, USA). The plasmids were electrophoresed in 1% agarose gel in 1x TAE buffer along with 1 kb DNA marker. The CAV plasmid DNA was isolated for each clone separately. The isolated recombinant plasmid was quantified by the spectrophotometric method at a wavelength of 260 nm. The quantified, cloned CAV plasmid DNA was used to assess the sensitivity for each CAV gene separately.

Calculation of no. of molecules in a known plasmid DNA concentration

1 µg/mL of 1000 bp DNA = 9.1×10^{11} molecules/mL

$$X \text{ µg/mL of Y bp DNA} = \frac{X \times 1000 \times 9.1 \times 10^{11} \text{ molecules/mL}}{Y}$$

Where,

X = concentration of plasmid DNA

Y = size of the plasmid along with the insert.

Primer designing. For each gene of CAV, two sets of primers were designed by using Fast PCR software (Primer Digital Ltd., primerdigital.com) based on the sequences of the CAV strain Cux-1 (Accession No: M 55918) and synthesized (Table 2). The designed primers were also aligned with the available Genbank data to assess its suitability in detecting field samples.

Standardization of PCR. The PCR was carried out in 25 µL reaction volumes using Red dye PCR master mix (GeNei, Bangalore, India) and the reaction mixture was set in a PTC-100 thermal cycler (M.J Research, U.S.A). The PCR products were run in 1% agarose gels prepared in 1x TAE buffer. Five µL of the amplified product were mixed with 2 µL of loading dye charged into the wells along with 100 bp DNA ladder (Biogene, USA). The electrophoresis was done at 90 volts for 45 min. The gel was viewed under UV-transilluminator and documented in gel documentation system (Bio Rad, USA).

Sensitivity assessment of PCR for CAV genes. The cloned and quantified CAV plasmid DNA was serially diluted tenfold in sterile nuclease free water and sensitivity of PCR was determined. The sensitivity of PCR to all the sets of primers was assessed separately for

Table 1. Details of published primers used with PCR cycle conditions (IWATA et al., 1998)

Gene name	Primer ID	Primer sequence	Length	PCR cycle conditions -Temperature (^{0}C) and time (min)					Expected smplicon size in bp
				Initial denatu-ration	Denatu-ration	Annealing	Extension	Final extension	
VP1	VP1.1	FP 5' TGT AAT ATG GAA AGA CGA GC 3' RP 5' CTT TTC AGG GCT GCG TCC CG 3'	20 mer 20 mer	94 for 1	94 for 1	60 for 1	72 for 2	72 for 7	1360
VP2	VP2.1	FP 5'GGG TGG ATG CAC GGG AAC GG 3' RP 5' GCC TTA CAC TAT ACG TAC CG 3'	20 mer 20 mer	94 for 2	94 for 1	55 for 1	72 for 1	72 for 5	660
VP3	VP3.1	FP 5' TTT CAA ATG AAC GCT CTC CA 3' RP 5' TCT TAC AGT CTT ATA CAC CT 3'	20 mer 20 mer	94 for 2	94 for 1	55 for 1	72 for 1	72 for 5	374

* the different PCR cycles were repeated for 29 times

each gene of CAV. The highest dilution of the plasmid DNA showing a visible band was taken as the detection limit.

Results

CAV Plasmid DNA isolation and quantification: The recombinant CAV plasmid (pCR4 -CAV-VP1 and pCR4 -CAV-VP2&3) available from the Department of Animal Biotechnology, Madras Veterinary College, was propagated and isolated. The isolated recombinant plasmids were observed in 1% agarose gel (Fig.1). The isolated recombinant plasmids were quantified, and the concentration of the plasmid pCR4 -CAV-VP1 was found to be 50 µg/ mL, and of plasmid pCR4 -CAV-VP2&3 it was also 50 µg/ mL. The quantified plasmids were stored at -20 ˚C for further use.

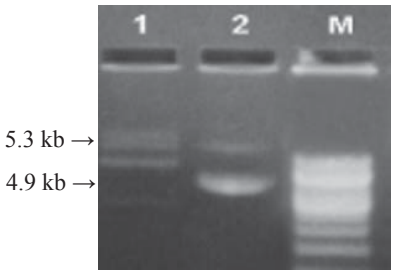

Fig. 1. CAV genes cloned plasmids run in 1% agarose gel. Lane 1: pCR4- TOPO CAV-VP1 recombinant plasmid; Lane 2: pCR4- TOPO CAV-VP2&3 recombinant plasmid; Lane M:1 kb DNA marker

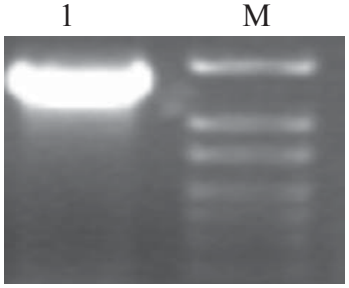

Fig. 2a. CAV VP1 gene amplicon using published primers. Lane 1: 1360 bp VP1 amplicon; Lane M: 100 bp DNA ladder

PCR primers

Published primers. PCR amplified products using published primers were 1360 bp, 660 bp and 374 bp for VP1, VP2 and VP3, respectively and were shown in 1% agarose gel (Fig. 2a, b, c).

Table 2. Sequences of designed primers used for assessing the sensitivity of PCR for the three CAV genes with cycle conditions

Gene name	Primer ID	Primer sequence	Length	PCR cycle conditions -Temperature (^{0}C) and time (min/sec)					Expected amplicon size in bp
				Initial denatu-ration	Denatu-ration	Anneal-ing	Extensi-on	Final extensi-on	
VP1	VP1.2	FP 5' ATG GCA AGA CGAGCT CGC AGA 3' RP 5' CCC CCC CTT TTC AGG GCT GCG 3'	21 mer 21 mer	94 for 2	94 for 45	65 for 1	72 for 1	72 for 7	1327
VP1	VP1.3	FP5' TGG CAA GAC GAG CTC GCA GAC C 3' RP 5' CCC AGT ACA TGG TGC TGT TCG 3'	22 mer 21 mer	94 for 1	94 for 45	62 for 1	72 for 1	72 for 7	1335
VP2	VP2.2	FP 5' ATG CAC GGG AAC GGC GGA CAA3' RP 5' TCA CAC TAT ACG TAC CGG GGC 3'	21 mer 21 mer	94 for 2	94 for 1	63 for 1	72 for 1	72 for 5	620
VP2	VP2.3	FP 5' ATG CAC GGG AAC GGC GGA CAA 3' RP 5' ACT ATA CGT ACC GGG GCG GGG GTT3'	21 mer 24 mer	94 for 2	94 for 1	64 for 1	72 for 1	72 for 5	608
VP3	VP3.2	FP 5' CAT GAA GCT CTC CAA GAA GAT ACT 3' RP 5' TTA CAG TCT TAT ACG CCT TTT TGC 3'	24 mer 24 mer	94 for 2	94 for 1	59 for 1	72 for 1	72 for 5	308
VP3	VP3.3	FP 5' TTT CAA ATG AAC GCT CTC CA 3' RP 5' TCT TAC AGT CTT ATA CAC CT 3'	20 mer 20 mer	94 for 2	94 for 1	62 for 1	72 for 1	72 for 5	334

* the different PCR cycles were repeated for 29 times

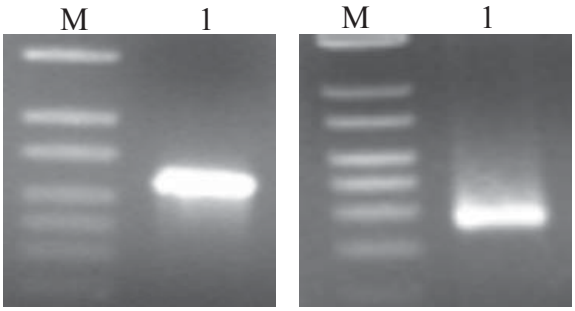

Fig. 2b and c. CAV VP2&3 gene amplicons using published primers
2b: Lane M: 100 bp DNA ladder; Lane 1: 660 bp VP2 amplicon
2c: Lane M: 100 bp DNA ladder Lane 1: 374 bp VP3 amplicon

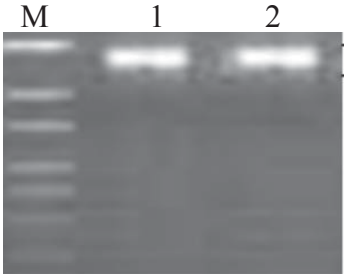

Fig. 3a. CAV VP1 gene amplicons using VP1.2, VP1.3 primers. Line M: 100 bp DNA ladder; Lane 1:1327 bp VP1 amplicon with VP1.2 primers; Lane 2: 1335 bp VP1 amplicon with VP1.3 primers

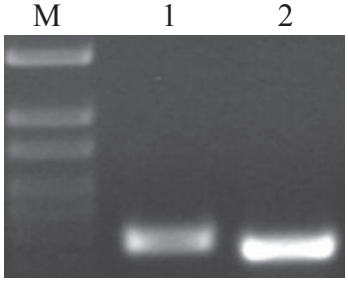

Fig. 3b. CAV VP2 gene amplicons using VP2.2, VP2.3 primers. Lane M: 100 bp DNA ladder; Lane 1: 620 bp VP2 amplicon with VP2.2 primers; Lane 2: 608 bp VP2 amplicon with VP2.3 primers

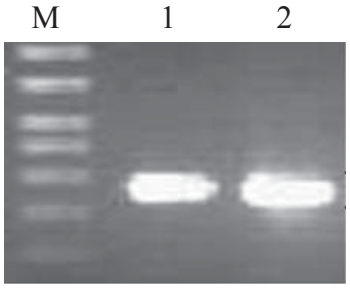

Fig. 3c. CAV VP3 gene amplicons using VP3.2, VP3.3 primers. Lane M: 100 bp DNA ladder; Lane 1: 308 bp VP3 amplicon with VP3.2 primers; Lane 2: 334 bp VP3 amplicon with VP3.3 primers

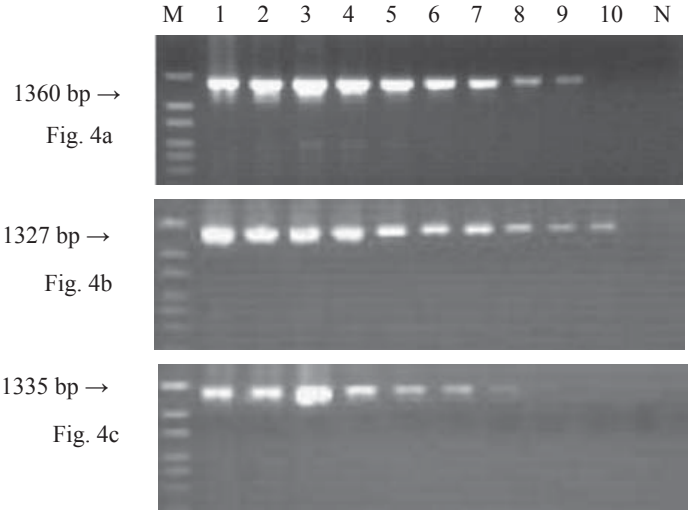

Fig. 4a, b, c. Sensitivity of PCR with VP1.1, 1.2 and 1.3 primers in CAV. detection. Lane M: 100 bp DNA ladder; Lane 1: Neat CAV plasmid DNA with VP1 gene; Lane 2-10: 10^{-1} to 10^{-9} dilutions of plasmid DNA (pCR4- TOPO CAV-VP1) - Fig. 4a; Lane 2-11: 10^{-1} to 10^{-10} dilutions of plasmid DNA (pCR4-TOPO CAV-VP1) - Fig. 4b; Lane 2-10: 10^{-1} to 10^{-9} dilutions of plasmid DNA (pCR4-TOPO CAV-VP1) - Fig. 4c; Lane N: Negative control

Designed primers. Two sets of primers for each CAV gene were designed through Fast PCR software viz. VP1.2, VP1.3 for VP1 gene, VP2.2, VP2.3 for VP2 gene and VP3.2, VP3.3 for VP3 gene. The designed primer sequences are given in Table 2. The alignment of the designed primers with the available Genbank sequences were found to be matching.

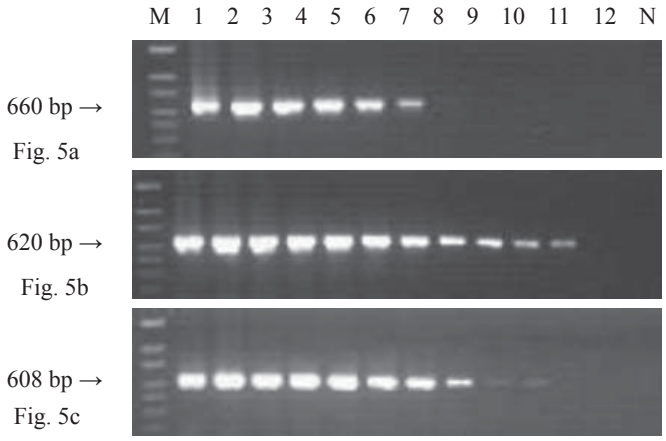

Fig. 5a, b, c. Sensitivity of PCR with VP 2.1, 2.2 and 2.3 primers in CAV detection. Lane M: 100 bp DNA ladder; Lane 1: Neat CAV plasmid DNA with VP2 gene, Lane 2-10: 10^{-1} to 10^{-9} dilutions of plasmid DNA (pCR4- TO; O CAV-VP2&3)- Fig. 5a; Lane 2-12: 10^{-1} to 10^{-11} dilutions of plasmid DNA (pCR4-TOPO CAV-VP2&3)- Fig. 5b; Lane 2-12: 10^{-1} to 10^{-11} dilutions of plasmid DNA (pCR4-TOPO CAV-VP2&3) -Fig 5c; Lane N: Negative control

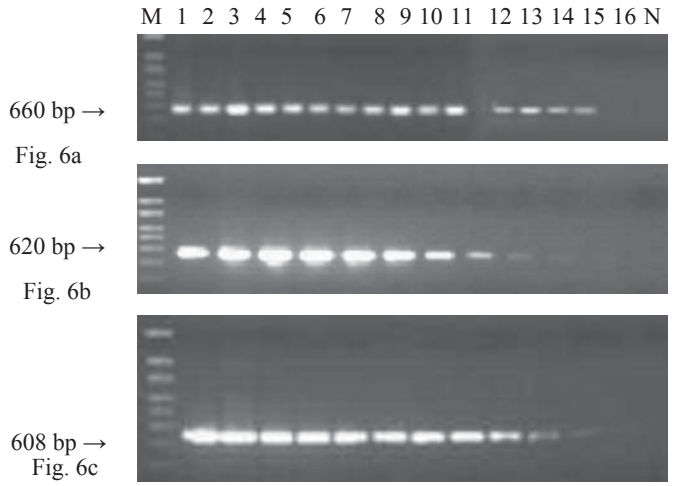

Fig. 6a, b and c. Sensitivity of PCR with VP3.1, 3.2 and 3.3 primers in CAV detection. Lane M: 100 bp DNA ladder; Lane 1: Neat CAV plasmid DNA with VP3 gene; Lane 2-16: 10^{-1} to 10^{-15} dilutions of plasmid DNA (pCR4-TOPO CAV-VP2&3) - Fig. 6a; Lane 2-11: 10^{-1} to 10^{-10} dilutions of plasmid DNA (pCR4-TOPO CAV-VP2&3) - Fig. 6b; Lane 2-11: 10^{-1} to 10^{-10} dilutions of plasmid DNA (pCR4-TOPO CAV-VP2&3) - Fig. 6c; Lane N: Negative control

Standardization of PCR with designed primers. The PCR cycle conditions were standardized for VP1.2 and VP1.3, VP2.2 and VP2.3 and VP3.2 and VP3.3 primers as given Table 2. The expected amplicon size of 1327 bp and 1335 bp, 620 bp and 608 bp and 308 bp and 334 bp, respectively was noticed in the 1.5% agarose gel as a clear single band (Fig. 3a, b, c).

Sensitivity of PCR with published and designed primers. The sensitivity of PCR reaction in CAV gene detection was assessed by using a 10 fold serially diluted CAV plasmid DNA template with the nine sets of primers for the three CAV genes.

Sensitivity of PCR for VP1 gene. The highest dilution of the plasmid DNA template (CAV VP1) showing a visible band in PCR was taken as the detection limit. The sensitivity with VP1.1 primers appeared in 10^{-8} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.5 fg/µL or 8.6×10^4 molecules/mL (Fig. 4a). With VP1.2 primers, the PCR amplicon was noticed up to 10^{-9} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.05 fg/µL or 8.6×10^3 molecules/mL (Fig. 4b). With VP1.3 primer, the PCR amplicon was noticed up to 10^{-6} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.05 pg/ µL or 8.6×10^6 molecules/mL (Fig. 4c).

Sensitivity of PCR for VP2 gene. With VP2.1 primers, the PCR amplicon was noticed up to 10^{-5} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.5 pg/µL or 9.9×10^7 molecules/mL (Fig. 5a). With VP2.2 primers, the PCR amplicon was noticed up to 10^{-10} dilution and the concentration of the plasmid DNA template at this dilution was found to be 5 ag/ µL or 9.9×10^2 molecules/ mL (Fig. 5b). With VP2.3 primers, the PCR amplicon was noticed up to 10^{-9} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.05 fg/µL or 9.9×10^3 molecules/mL (Fig. 5c).

Sensitivity of PCR for VP3 gene. With VP3.1 primers, the PCR amplicon was noticed up to 10^{-14} dilution and the concentration of the plasmid DNA template at this dilution was found to be 5×10^{-4} ag/µL or 10.5×10^{-2} molecules/ mL (Fig. 6a). With VP3.2 primers, the PCR amplicon was noticed up to 10^{-9} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.05 fg/µL or 10.5×10^3 molecules/mL (Fig. 6b). With VP3.3 primers, the PCR amplicon was noticed up to 10^{-9} dilution and the concentration of the plasmid DNA template at this dilution was found to be 0.05 fg/µL or 10.5×10^3 molecules/mL (Fig. 6c).

Sensitivity of PCR for the three genes. For the CAV VP1 gene, of the three primer sets assessed for sensitivity, VP1.2 primers were the most sensitive and the concentration of the plasmid DNA template was 0.05 fg/µL or 8.6×10^3 molecules/ mL. For VP2 gene, VP2.2 primers were the most sensitive of the three primer sets and the concentration of the plasmid DNA template was 5 ag/ µL or 9.9×10^2 molecules/ mL. For the VP3 gene, VP3.1

primers were the most sensitive of the three primer sets tested and the concentration of the plasmid DNA template was 5×10^{-4} ag/µL or 10.5×10^{-2} molecules/mL.

Discussion

CAV is prevalent worldwide wherever poultry is reared in large numbers, after the first report in Japan (YUASA et al., 1979). Its confirmatory diagnosis is made conventionally by virus isolation in specific pathogen-free (SPF) chicks or SPF embryos or cell cultures and serological tests viz. Indirect Immuno Fluorescent Antibody Test (IFAT), Enzyme Linked Immuno Sorbent Assay (ELISA) and virus neutralization test (SENTHILKUMAR et al., 2003). PCR is as good as virus isolation, and can detect one copy of CAV DNA. It has gained importance by giving a faster result and more sensitivity in detecting not only fastidious viral pathogens but also subclinical infections.

In this work, cloned CAV DNA templates were used for PCR sensitivity assessment due to insufficient PCR positive CAV tissue DNA but they are also preferable in terms of purity. This could be a suitable model for DNA field samples. The recombinant plasmid with CAV genes helps to regenerate the infectious virus and further biological characterization as demonstrated by BROWN et al. (2000). They cloned the full length CAV genome in plasmid vector pGEM-4Z and transfected it into *in vitro* cultured cells, which were used to study viral gene functions and CAV pathogenesis. But in this study, the cloned CAV plasmid DNA was used for PCR sensitivity assessment and it would also be possible to produce an infectious clone of the virus, when the genome of CAV genes are available as cloned plasmid.

Understanding the importance of each gene of CAV in diagnosis and also considering the need for PCR sensitivity assessment in amplifying complete ORF of each gene of CAV, the present study was carried out. Many workers have used primers to amplify a complete/partial VP1 gene portion (TODD et al., 1992; OLUWAYELU et al., 2005) and for complete gene amplification of VP2 and VP3 genes (DANTAS et al., 2007). Hence, the present study was designed to use a total of nine sets of primers with 3 sets of primer for each gene of CAV comprising one set of published primers and two sets of designed primers. The Fast PCR programme used for primer designing was found to be good. The sequence used in primer designing was the European strain - Cux-1 of CAV (Accession No: M 55918). As CAV has only one serotype (McNULTY, 1991) and Cux 1 is the reference strain of CAV, the use of the genome sequence in primer designing was quite appropriate. The primers were designed in such a way that the ORF of each was involved.

Many workers have conducted PCR sensitivity assessment for VP1 gene primers. In this study, among the three primer sets used for VP1 gene, VP1.2 primer set was the most sensitive. The PCR amplicon (1327 bp) was observed up to 10^{-9} dilution with a plasmid

DNA template concentration of 0.05 fg/µL and the equivalent DNA copy number was 8.6 $\times 10^3$ molecules/mL.

The earlier report of THAM and STANISLAWEK (1992) stated that their PCR assay detected a single CAV infected MSB 1 cell and at least 100 fg of CAV replicative form (RF) DNA extracted from infected MSB 1 cells, and they used a modified assay which could increase the sensitivity to 100 fold, i.e. at 1 fg of DNA, which they explained by the use of different thermal cyclers. They proved that PCR is a useful technique for CAV DNA detection in field samples. Our study also demonstrates that the field CAV detection limit could be extended further to the level of detecting subclinical infection due to the use of the most sensitive primer.

In another study, TODD et al. (1992) showed that PCR amplification of a partial VP1 gene (675 bp) was possible with 0.1 fg of the DNA template, whereas in the present work, a full ORF of VP1 gene was amplified with good sensitivity with the designed primer VP1.2.

The use of a cloned CAV DNA template for the sensitivity of PCR was reported previously by using serial 10-fold dilutions of plasmid pCAA -3 DNA containing CAV inserts (OLUWAYELU et al., 2005). It was carried out to amplify a fragment of the CAV VP1 gene (733 bp) with the designed primers. They used a partial VP1 gene for amplification and obtained sensitivity of 10^{-11}, but in this study, with the complete VP1 gene amplifying primers, sensitivity was obtained up to 10^{-9} dilution with a plasmid DNA concentration of 0.05 fg/µL.

VP2, a 28 kDa dual specificity protein phosphate (DSP), is a multifunctional protein with a non-structural role in virus infection and replication. Expression studies have shown that co-expression of both VP1 and VP2 is required for the generation of recombinant proteins capable of inducing neutralizing antibodies (NOTEBORN et al., 1998). Thus, VP2 may also have a role as a scaffolding protein during virion assembly and hence the effect of VP2 mutations that alter protein secondary structure could be used to slow down virus assembling. Detection of the VP2 gene also has importance in understanding its functions.

Among the three sets of primers used in this study for assessing the sensitivity of the VP2 gene, the designed primer VP2.2 was found to be most sensitive. Its sensitivity was noted up to 10^{-10} dilution and the concentration of the plasmid DNA was 5 ag/ µL or 9.9 x 10^2 molecules/ mL. The sensitivity of PCR for the VP2 gene of CAV has not been reported previously. This was the first attempt to study the sensitivity of the VP2 gene of CAV using a plasmid cloned CAV VP2 template.

Among the three sets of primers used in this study for assessing the sensitivity of the VP3 gene, the published primer VP3.1 was found to be most sensitive. The sensitivity was noticed up to 10^{-14} dilution and the concentration of the plasmid DNA was 5 $\times 10^{-4}$ ag/µL

or 10.5 $\times 10^{-2}$ molecules/mL. The sensitivity assessment of PCR for the VP3 gene of CAV has also not been reported previously, although the PCR assay for the CAV VP3 gene has been standardized and validated (NOGUEIRA et al., 2005) and was still being used earlier by scientists (IWATA et al., 1998). This was also the first attempt to study the sensitivity of a cloned VP3 gene as a DNA template. Although there are some nucleotide variations among CAV isolates, the most sensitive primers could be used for diagnostic application. The findings of this study may be very well applied for diagnostic, sequencing, and cloning and expression purposes.

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SAŽETAK

Matrice gena virusa anemije pilića klonirane u plazmidnoj DNA (pCR4-CAV-VP1 te pCR4-CAV-VP2 i 3) bile su rabljene radi razvijanja osjetljive lančane reakcije polimerazom (PCR) za njihovo dokazivanje. Za procjenu osjetljivosti PCR-a bilo je rabljeno devet kompleta početnica među kojima je bio jedan komplet već objavljenih i dva kompleta sintetiziranih početnica za svaki gen virusa anemije pilića, odnosno za VP1, VP2 i VP3. Uvjeti PCR-a za umnožavanje bili su standardizirani sa sintetiziranim početnicama kako bi se dobio pojedinačni specifični umnožak za svaki gen. Osjetljivost PCR-a bila je procijenjena na osnovi deseterostrukih serijskih razrjeđenja kloniranih plazmidnih matrica u reakciji rabljenih u svakom kompletu početnica za svaki gen virusa. Najveće razrjeđenje plazmidne DNA virusa koje je pokazivalo jasno vidljiv umnožak bilo je uzeto kao krajnja mogućnost dokaza. Rezultati su pokazali da je proizvedena početnica VP1.2 bila osjetljivija za gen VP1, a koncentracija plazmidne DNA iznosila je 0,05 fg/μL ili 8,6 $\times 10^3$ molekula/mL. Početnica VP2.2 bila je osjetljivija za gen VP2, a koncentracija plazmida bila je 5 ag/μL ili 9,9 $\times 10^2$ molekula/mL. U slučaju VP3, objavljena početnica VP3.1 pokazala se osjetljivijom za gen VP3, a koncentracija plazmida iznosila je 5 $\times 10^{-4}$ ag/μL ili 10,5 $\times 10^{-2}$ molekula/mL. Nalazi ovog istraživanja mogu biti korisni u dijagnostici, sekvencioniranju, kloniranju i ekspresiji.

Ključne riječi: virus anemije pilića, lančana reakcija polimerazom, početnice, umnožak