

# The Consequence of a Single Nucleotide Substitution on the Molecular Diagnosis of the Chicken Anemia Virus

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## ABSTRACT

While genomic variations, including single nucleotide polymorphism (SNP) are expected and common for RNA viruses, their occurrence is anticipated at a fairly low frequency for Chicken Anemia Virus (CAV), as it contains a conserved DNA genome. The present report demonstrate that in 4/80 CAV field isolates one nucleotide substitution, from G to A, located in the middle of the real-time probe was responsible for false-negative real-time PCR amplification results. This finding emphasizes the need of awareness to harmful mismatches that occur even in conserved genomes, and the need for periodical verification of amplification primers and probes according to the clinical picture in the field.

**Keywords:** Chickens; Chicken Anemia Virus; Molecular Diagnosis; SNP

## INTRODUCTION

Chicken anemia virus (CAV) is ubiquitous with a worldwide distribution having a considerable economic impact due to its ability to cause clinical morbidity, increased mortality, but also sub-clinical infections and immune-suppression (1). CAV belongs to the family *Circoviridae*, the *Gyrovirus* genus and is a non-enveloped virus with a negative sense single-stranded circular DNA genome and is considered a conserved virus of one serotype.

The viral genome consists of 2.3 kb with three partially or completely overlapping ORFs (open reading frames). ORF3 (1,347 bp) encodes the major viral structural protein VP1 (52 kDa) and partially overlaps with ORF1 (648 bp), VP2 which encodes the 24 kDa protein VP2, a scaffolding protein. ORF3 (VP3) encodes a non-structural protein named apoptin (13.6 kDa) for its ability to cause apoptosis. VP1 is the target of neutralizing antibodies, while VP2 is important to fold VP1 in the proper format. CAV sequence variability consisted of less than 6% at the amino acid level, especially at the VP1 hypervariable region, amino acids 139-151 of VP1 (2, 3)

and also at the carboxy-terminus of VP2 and VP3 (4). The present phylogenetic classification of sequenced worldwide CAV isolates is not supported by any biological distinction; therefore, the significance of the phylogenetic grouping is still rather vague (5).

The VP2 protein was demonstrated in several Bangladesh and American CAV isolates to be the most conserved among the three CAV proteins, showing only 1.4% diversity, compared to about 4% and 2.2% for VP1 and VP3, respectively (6, 7). To reduce possible genomic mismatches due to genetic variability between field strains and to consolidate the diagnostic assay, the VP2 gene was adopted at our laboratory for molecular diagnosis by end-point PCR according to Imai *et al.* (8) and by real-time amplification based on a plasmid in which the end-point PCR amplicon was inserted, which served as the positive control DNA and as the standard for quantification (9).

While the PCR-based detection of CAV is the most widely applied diagnostic test, due to its straightforward advantages in relative easiness of fast performance and sensitivity, minor genomic changes might impede virus detec-

tion. While genomic variations, including single nucleotide polymorphisms (SNP) are expected and common for RNA viruses, their occurrence and detrimental influence on diagnosis is unanticipated for CAV, as the virus is considered genetically conserved DNA genome. The evolutionary rate of DNA genomes is  $10^{-3}$  lower than RNA genomes,  $10^{-6}$  to  $10^{-8}$ /base/generation compared to  $10^{-3}$  to  $10^{-5}$ /base/generation, for DNA and RNA viruses, respectively (10). Strain-specific primers which discriminate between CAV strains have been reported previously (11).

In this communication we describe an uncommon SNP that occurred in the genomic area encompassing the probe used in the real-time PCR (2).

## MATERIALS AND METHODS

### Clinical samples

The study included 80 commercial chicken flocks of various ages and type that were submitted for molecular diagnosis, between January 2013 to March 2014 and was positive for CAV by end-point PCR (12). Basically, DNA was purified from samples of spleen and liver as a pool and from 10 pieces of 5 feather shafts, as a second pool. Similar organs from 3 chickens were pooled for DNA purification and used as one DNA sample.

### DNA purification and end-point PCR

DNA was purified using Maxwell 16 Tissue DNA Cat. # 1030 kit, (Promega, Mad. WI, U.S.A.), according to the manufacturer instruction, and amplified for CAV (1). The PCR cloned amplicon, was used as positive control.

### Primers and probes of real-time PCR

The cloned CAV amplicon, obtained from a local CAV isolate was the basis of the following original first set of real-time PCR primers and probes:

Forward, 5' – CCGCGCTAAGATCTGCAACTG,  
Reverse, 5' – GAGGGAGGCTTGGGTTGATC  
Probe 5' FAM/3' BHQ-1 CGGACAATTCCGAAA  
GCACTGGTTTCA.

Following the identification of the SNP, a second pair of primers and probe was designed, matching with the Cux-1 CAV prototype (Acc. No. M55918):

Forward, 5' – CCA CGCTAAGATCTGCAACTG,  
Reverse, 5' – GAGGGAGGCTTGGGTTGATC

Probe 5' FAM/3' BHQ-1 CGGACAATTCAGAAAG/  
ACACTGGTTTCA.

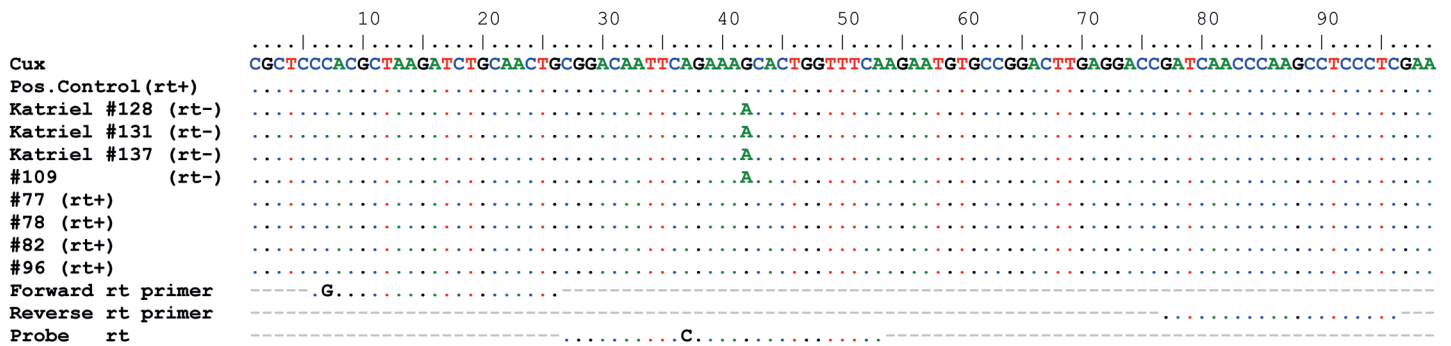
A sample of 2  $\mu$ l of the extracted DNA, according to the kit instructions, was used in the real-time PCR assay, as described (9).

## RESULTS AND DISCUSSION

In November 2013 three broiler flocks derived from the same broiler breeding flock that showed typical clinical signs of CAV and immunosuppression-related morbidity, were submitted for CAV diagnosis. About 4% mortality was recorded on days 13–26 of age, accompanied by severe hypocellularity of the bone marrow, thymus and spleen atrophy with lymphocytic depletion, bone marrow lesions, foot inflammatory lesions, necrosis, and perihepatitis/pericarditis in about 95% of the birds that were examined post-mortem.

The real-time amplification of these samples was negative for CAV, therefore the samples were amplified by the end-point PCR assay. As suspected, the presence of CAV was demonstrated. After elimination of technical factors, a mismatch between the CAV genome and the real-time PCR primers and probes was suspected. To evaluate whether additional false negative diagnostic cases occurred since January 2013, all CAV-negative cases (n=77) by real-time PCR, amplified with the first primer and probe set, were retested by end-point PCR. Only one out of 77 flocks was found false-negative by real-time PCR. In total, the false-negative samples were 5% out of 80 flocks, that is 4 flocks.

We then compared the end-point PCR amplicon sequences of the 4 flocks that were negative by the real-time PCR (Katriel #128, Katriel #131, Katriel #137 and #109), 4 reactive flocks (#77, #78, #82 and #96), as well as the end-point PCR amplicon of the local isolate (positive control), the CAV Cux-1 prototype amplicon and the real-time PCR first set of primers and probe (Fig. 1). As reflected in the sequence alignment, the non-reactive 4 flocks differed by one nucleotide substitution, from G to A, located in the middle of the real-time probe. The presence of an additional SNP, derived from the local isolate sequence that was present on the probe sequence allowed amplification, however, the addition of a second SNP present on the forward primer, was detrimental to amplification. Furthermore, to ascertain the SNP contribution, a second set of CAV primers and probes were synthesized according to the Cux-1 sequence, i.e., without the local isolate



**Figure 1.** Sequence alignment of 4 reactive and 4 non-reactive flocks by CAV real-time PCR with the first set of primers and probe, performed with Mega program ver. 5.0.

SNP. By employing the second set of primers all samples were amplified by real-time PCR, indicating the importance of the novel G to A substitution in the probe region.

The present findings confirm many previous studies reporting the effect of various sequence mismatches, demonstrating more pronounced effects on amplification caused by mismatch-modified probe than those of mismatch-modified primers. Specifically, Süß et al. (13) showed that probe mismatches have a double weighted effect compared to primer mismatches.

In summary, the present case emphasizes the need of awareness to detrimental mismatches that occur even in conserved genomes, leading to the need of periodical verification of amplification primers and probes, according to the clinical picture in the field.

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