



Survey on the Prevalence of Chicken Anaemia Virus among Birds of Various Production types in Bulgaria



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Abstract

A total of 378 thymus samples from chickens of various production types-growing layer hens, broilers and backyard birds were assayed by Polymerase Chain Reaction (PCR) for detection of Chicken Anaemia Virus (CAV) Desoxyribo Nucleic Acid (DNA). The samples have been collected and examined in the period 2012-2017. For this study, 140 thymus samples from growing layer hens, 202 from broiler chickens and 36 from backyard birds were used. The chickens were at the age of 10days to 17weeks and originated from 15 regions of Bulgaria. In this study, a total of 86 poultry flocks including 16 growing layer hens, 60 broiler and 10backyard chicken flocks were surveyed as 65 commercial poultry farms, small farms and backyards from all over the country were included.

The PCR assay determined the presence of CAV DNA in 25 chickens aged up to 16 weeks originating from 7 regions of Bulgaria. The results revealed that 5 (3.57%) of 140 growing layer hens and 20 (9.9%)of 202 broilers examined were positive for CAV, while the virus was not confirmed in the 36 backyard birds. The overall prevalence of CAV was 6.61% and ranged between 1.75% and 100% in the various regions of Bulgaria. CAV was found in 11 (12.79%) of 86 poultry flocks surveyed, including 3 growing layer hens and 8 broiler chicken flocks. Furthermore, correlation between the prevalence of CAV in flocks and the age of chickens in them was found, which consisted in decreasing the prevalence of CAV with increasing the age of birds. The corpses of only three of the broilers positive for CAV were with anaemia. These findings not only demonstrate that CAV is present in the country, but they also represent the first detection of CAV DNA by PCR in broiler chickens in Bulgaria.

Keywords: Chicken anaemia virus; Prevalence; Growing layer hens; Broilers; Backyard birds; Polimerase chain reaction

Abbreviations: CAV: Chicken Anaemia Virus; PCR: Polymerase Chain Reaction; CIA: Chicken Infectious Anaemia; DNA: Desoxyribo Nucleic Acid; ORFs: Open Reading Frames

Introduction

CAV was first isolated and described by Yuasa and colleagues in Japan in 1979. The virus is a small non enveloped icosahedral DNA virus with diameter 19,1-26,5nm [1,2] and belongs to the genus Gyrovirus of the family Circoviridae [3]. CAV has a closed circular negative single-stranded DNA genome of approximately 2.3kb [4,5] which consists of three partially overlapping Open Reading Frames (ORFs) encoding three viral proteins: VP1, VP2 and VP3[6,7]. VP1 (51,6kDa) is the major viral structural protein [9]. VP2 (24kDa) not only possesses dual-specificity phosphatase activity but also acts as scaffolding protein during virion assembly [8,9]. Lastely, VP3 (13,6kDa), also called apoptin, is the smallest protein of CAV which exhibits apoptosis-induced activity in transformed celllines [10]. CAV replicates in the precursor T-lymphocytes and in the haemocytoblasts of the bone marrow, causing thymus atrophy and anaemia [11,12].

Chicken Infectious Anemia (CIA) is characterized by increased mortality, severe anaemia, growth retardation, intramuscular and subcutaneous haemorrhages, yellowish bone marrow, and severe atrophy of the thymus in young chicks [13-15]. CAV causes clinical disease in transovarially infected chickens not protected by maternal antibodies in the first 2weeks of life [16]. In chickens of more than two weeks CAV usually causes subclinical disease [17], which leads to immune suppression resulting in serious secondary infections caused by other pathogens and even decreasing the efficacy of vaccines after immunization [18]. Maternal antibodies prevent clinical disease but do not prevent infection, transmission of the virus, or immune suppression [19]. The clinical disease is rare today because of the wide spread practice of vaccinating breeder flocks, but the subclinical form of the disease is ubiquitous [20]. In this study, the results of the examinations by PCR for detection

of CAV DNA in birds of different production types in Bulgaria are presented.

Materials and methods

Samples collection

A total of 378 thymus samples from dead birds, including 140 from growing layer hens, 202 from broilers and 36 from backyard chickens have been collected and examined during the period of 2012 to 2017. The birds aged between 10 days and 17 weeks originated from 65 commercial poultry farms, small farms and backyards located in 15 regions of Bulgaria: Blagoevgrad, Varna, Vidin, Vratsa, Dobrich, Kyustendil, Kurdzhali, Lovech, Montana, Plovdiv, Razgrad, Russe, Stara Zagora, Targovishte and Shumen. A total of 86 poultry flocks including 16 growing layer hens, 60 broiler and 10 backyard chicken flocks were surveyed.

DNA extraction and PCR assay

According to the manufacturer's instructions, CAV DNA was extracted from thymus samples of the 378 birds using the Tissue and Cell Genomic DNA Mini Kit (Guangzhou Geneshun Biotech, China).

Two sets of oligonucleotide primers were used for amplification of CAV DNA: primers S.1.1.: 5'-AATGAACGCTCTCCAAGAAG-3' and S.1.2.: 5'-AGCGGATAGTCATAGTAGAT-3' [21], which comprised a fragment of 583 bp (positions 485-1067) of the published genome of the reference strain of Cuxhaven-1, as well as primers CAV-VP1F: 5'-GAC TGT AAG ATG GCA AGA CGA GCT C-3' and CAV-VP1R: 5'-GGC TGA AGG ATC CCT CAT TC-3', flanking a 675 bp fragment which encodes most of the N-terminal half of the putative gene for the CAV capsid protein [22]. The working concentration of each primer was 10 pmol/μL.

The PCR assay was performed as described previously [21,22], but in a total volume of 25 μL (12.5 μL master mix, 2 μL of primer S.1.1. or CAV-VP1F, 2 μL of primer S.1.2. or CAV-VP1R, 2 μL of target DNA and 6.5 μL nuclease-free water), in automatic thermo cycler (QB-96, LKB or TECHNE TC-412). The cycling profile of amplification performed with a pair of primers S.1.1./S.1.2. consisted in the following steps and conditions: one cycle of initial denaturation step at 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 56 °C for 2 min, and 74 °C for 2 min representing denaturation, annealing, and extension steps, respectively, and finally one cycle of final extension step at 74 °C for 10 min. The amplification with a pair of primers CAV-VP1F/R was carried out using the following cycling parameters: one cycle of initial denaturation step at 94 °C for 2 min followed by 50 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min representing denaturation, annealing, and extension steps, respectively, and finally one cycle of final extension step at 72 °C for 7 min.

The obtained PCR products were then analysed by electrophoresis on a 1.5% agarose gel as the expected band was visualized by staining with ethidium bromide. A 50-bp DNA ladder served as a size marker.

Results

Out of 378 thymus samples examined, specific PCR products (583 bp and 675 bp) Figure 1 were detected in 25 (6.61%) chickens of age up to 16 weeks originating from 7 regions of Bulgaria - Blagoevgrad, Kyustendil, Lovech, Plovdiv, Razgrad, Russe and Vratsa. CAV DNA was confirmed in 5 (3.57%) of 140 growing layer hens and in 20 (9.9%) of 202 broiler chickens tested, but not in the 36 backyard bird.

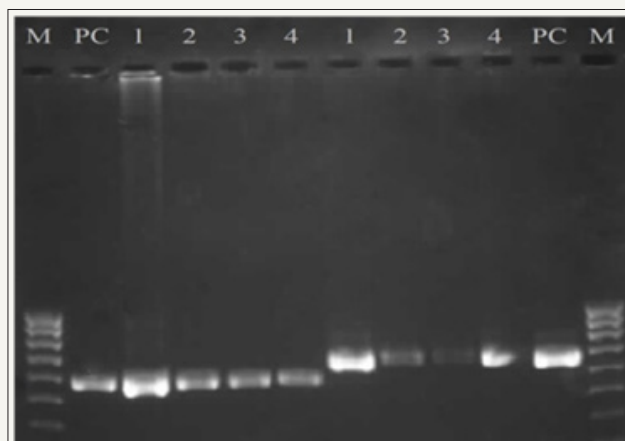


Figure 1: Agarose gel visualizing PCR products from amplification of thymus samples with a size of 583 bp and 675 bp, after performing of PCR with pairs of primers S.1.1./S.1.2. and CAV-VP1F/CAV-VP1R, respectively, with reference Positive Controls (PC) and four samples (1, 2, 3 and 4); Lanes M: Hyper Ladder 50 bp markers; lanes 1, 2, 3 and 4: positive thymus samples; Lanes PC: positive controls.

The overall prevalence of CAV was 6.61%, with 3.57% in growing layer hens and 9.9% in broiler chickens, and ranged between 1.75% and 100% in the various regions of Bulgaria. The highest prevalence was determined in Russe region (100%)

followed by Lovech (33.33%), Plovdiv (25%), Vratsa (20%), Blagoevgrad (16.67%), Razgrad (10.7%) and Kyustendil (1.75%) regions (Table 1). In addition, in the small broiler eco-farm in the village of Damyanitsa situated in Blagoevgrad region 8 (16.67%)

of 48 birds tested were found to be infected. CAV was detected by PCR in 11 (12.79%) out of 86 poultry flocks surveyed, including 3 growing layer hens and 8 broiler chicken flocks. Furthermore, correlation between prevalence of CAV in flocks and age of birds

in them was ascertained, which consisted in decreasing prevalence of virus with increasing age of birds (Table 2) The highest (100%) prevalence of CAV was recorded at the age between 21-33 and the lowest (10%) was recorded at 112 days in the flocks.

Table 1: Prevalence of CAV detected by PCR in birds of various production types.

№	Region in Bulgaria	Production type					
		growing layer hens		broilers		backyard birds	
		No. tested	No. positive / (%)	No. tested	No. positive / (%)	No. tested	No. positive / (%)
1	Blagoevgrad	-	-	48	8 (16.67%)	34	-
2	Dobrich	3	-	-	-	-	-
3	Kyustendil	114	2 (1.75%)	-	-	2	-
4	Kurdzhali	6	-	-	-	-	-
5	Lovech	2	-	3	1 (33.33%)	-	-
6	Montana	-	-	6	-	-	-
7	Plovdiv	4	1 (25 %)	-	-	-	-
8	Razgrad	-	-	28	3 (10.7%)	-	-
9	Russe	2	2 (100 %)	19	-	-	-
10	Shumen	-	-	24	-	-	-
11	Stara Zagora	-	-	37	-	-	-
12	Targovishte	-	-	15	-	-	-
13	Varna	4	-	2	-	-	-
14	Vidin	-	-	4	-	-	-
15	Vratsa	5	-	16	8 (20 %)	-	-
	Total	140	5(3.57%)	202	20 (9.90%)	36	-
	Overall prevalence 6.61%						

Table 2: Correlation between prevalence of CAV in poultry flocks and age of birds.

№ Flock	Farm in village/ town (Production type)	Region in Bulgaria	Capacity of the Farms/No. of Birds	Age (days)	No. tested by Pcr	No.of CAV Positive / (%positive)
1	Damyantsa-village(broilers)	Blagoevgrad	10 000	57	28	5(17.85%)
2	Damyantsa-village (broilers)	Blagoevgrad	10 000	96	20	3(15%)
3	Boboshevo - town (growing layer hens)	Kyustendil	6 900	112	20	2 (10%)
4	Debevo-village (broilers)	Lovech	60 000	33	1	1 (100%)
5	Tsalapitsa-village (growing layer hens)	Plovdiv	2100	39	3	1 (33.33%)
6	Beli Lom-village (broilers)	Razgrad	79 758	25	2	2 (100%)
7	Farm in Razgrad-	Razgrad	1 104000	45	6	1 (16.67%)

	town(broilers)					
8	Tchervenavoda-village	Russe	36415	29	2	2 (100%)
	(growing layer hens)					
9	Glavatsi-village (broilers)	Vratsa	20 000	31	2	2 (100%)
10	Kravoder-village (broilers)	Vratsa	10 000	21	5	5 (100%)
11	Kravoder-village (broilers)	Vratsa	10 000	36	2	1 (50%)
	Total				91	25(27.47%)

The corpses of only three of the broilers which were found to be positive for CAV both of them originating from Razgrad region and one from Lovech region showed anaemia.

Discussion

Although the CAV infection has been described in most countries with a developed chicken industry [23], the data on the incidence of CIA in Bulgaria are poor [24,25]. Considering this fact as well as the circumstance that the investigations conducting in some commercial and small poultry farms in different regions of the country showed characteristic of CIA signs such as anaemia, atrophy of the thymus, intramuscular and subcutaneous haemorrhages, the necessarily of monitoring of the disease in various regions of Bulgaria was suggested, which aimed to determine the importance of CAV for the country and to plan an adequate control strategy against this economically important avian pathogen.

PCR for in vitro amplification of specific DNA fragment has been emerging at the current stages a main reference high sensitive and specific rapid molecular assay for detection of CAV DNA [22]. The results of the examinations by PCR exhibited in this study revealed that 25(6.61%) chickens of age up to 16 weeks were positive for CAV as CAV DNA was detected in 5 (3.57%) of 140 growing layer hens and 20 (9.9%) of 202 broiler chickens tested. The percentage positive samples obtained from broilers is approximately three times lesser than that determined by Mohamed (2010) who confirmed CAV in 44 (26.6%) of 165 broilers, and two times higher than that found by Chowdhury and colleagues (2002) that detected CAV DNA in 5 (4%) of 125 broiler chickens with retarded growth aged 2-6 weeks. Bougiouklis and colleagues (2007) described a clinical case of CIA and virus DNA detection in naturally infected 12-day-old broilers. Other authors provide evidence for the occurrence of CIA in backyard chicken flocks. Barrios and colleagues (2009) confirmed CAV genome by nested PCR in 30% of the 20 flocks examined while the PCR assay performed by Oluwayelu and Todd (2008) determined the presense of CAV in 9 of 12 serum samples from apparently healthy backyard chickens. Previous study conducted in Bulgaria demonstrated confirming CAV by PCR in three of only seven 9-week-old growing layer hens tested [24], but there are still no data on detecting CAV DNA by PCR in broiler chicken flocks. Therefore, this survey provides an additional

information which elucidate the situation with the incidence of the disease not only in growing layer hens but also in broiler flocks in the country. Moreover, this study represents the first detection of CAV DNA by PCR in broilers in Bulgaria. Although CAV was not confirmed among backyard chickens previous data denote the high seroprevalence of the virus in poultry flocks of this production type in the country [24].

The correlation between prevalence of CAV in flocks and age of chickens in them which was found out in this study and consisted in decreasing prevalence of CAV with increasing age of birds, suggested developing seroconversion against CAV which occurs in parent flocks before or around point of lay and in broiler flocks at slaughter age, which is in accordance with the findings of other scientists. De Herdt and colleagues (2001) conducted serologic survey in unvaccinated broiler parent and broiler progeny flocks and found seroconversion against the virus in all parent flocks before or around point of lay and in 38% of the broiler flocks examined at slaughter age. Sommer and Cardona (2003) studied the dynamics of CAV infection in two broiler flocks from a commercial producer with detectable CAV antibodies at hatch which waned over the first three weeks of age. They found that at 35 days of age the virus was detectable by PCR in 16 of 20 chickens as 7 of 20 had antibodies to CAV and by 42 days of age the virus was detectable in 18 of 20 chickens and 18 of 20 had detectable antibodies.

The fact that only three of the 25 birds positive for CAV had anaemia revealed that it concerned to clinical disease only in them and to subclinical infection in the other 22 chickens, which is not surprising due to the high immune background of breeder flocks in Bulgaria. However, in the small eco-farm in the village of Damyanitsa situated in Blagoevgrad region the broiler chickens are bred according to the requirements of bio-production with out to be vaccinated and it is no accident that in this poultry farm 8 (16.67%) of 48 birds examined were infected.

Conclusion

This study provides novel information about the distribution of CAV and its prevalence among birds of various production types in Bulgaria. The exhibited results indicate that the CAV infection persists in growing layer hens and broilers as the prevalence of CAV

among broiler chickens is higher than that among growing layer hens. These findings not only denote that CAV is present in the country, but they also represent the first detection of CAV DNA by PCR in broiler chickens in Bulgaria. The small number of backyard chickens examined does not allow unconditional conclusions to be made but gives ground to be assumed that further studies could elucidate the situation with the occurrence of CIA in poultry flocks of this production type in Bulgaria [26-31].

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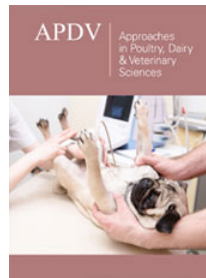
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