



ISOLATION AND PCR IDENTIFICATION OF CHICKEN ANAEMIA VIRUS INFECTION IN BULGARIA

K. B. SIMEONOV¹, R. T. PETROVA¹, B. I. GYUROV²,
R. D. PESHEV¹ & B. K. MITOV¹

¹Department of Virology; ²Department of Microbiology, National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria

Summary

Simeonov, K. B., R. T. Petrova, B. I. Gyurov, R. D. Peshev & B. K. Mitov, 2014. Isolation and PCR identification of chicken anaemia virus infection in Bulgaria. *Bulg. J. Vet. Med.*, **17**, No 4, 276–284.

A strain of chicken anaemia virus (CAV) was isolated on the lymphoblastoid cell line MSB1 from nine-week-old chickens, submitted to the laboratory for routine diagnostic investigation. The birds originated from a flock that exhibited increasing mortality within two weeks. Most of the investigated chickens showed gross pathological and histopathological lesions suggestive of both CAV and IBDV infections, such as paleness of the carcasses, subcutaneous and muscular haemorrhages throughout the body, bone marrow aplasia and thymic and bursal atrophy. After inoculation in the T-lymphoblastoid cell culture MSB1, initial signs of CPE, comprising enlargement of cells, growth slowdown and cell lysis were observed on the third passage. Investigation by PCR proved a presence of CAV DNA both in the thymuses of surveyed birds and in inoculated MSB1 cells. Together with CAV, coinfection with another infectious agent, suggestive for infectious bursal disease virus (IBDV) was detected. This is the first isolation of CAV in Bulgaria.

Key words: chicken anaemia virus, isolation, PCR

INTRODUCTION

Chicken anaemia virus (CAV), the only member of genus *Gyrovirus* of the Circoviridae family (Pringle, 1999), is a ubiquitous pathogen of chickens and has a worldwide distribution. In young chickens without maternal antibodies, CAV causes disease characterised by depression, anaemia/haemorrhagic syndrome, retarded growth and increased mortality (Schat, 2003). After about 3 weeks of age, im-

munocompetent chickens develop resistance to disease, although they can acquire infection and transmit the virus horizontally (Rosenberger & Cloud, 1989a, Toro *et al.*, 1997). CAV replicates in the haemocytoblasts of the bone marrow and in the precursor T-lymphocytes, causing anaemia and thymic atrophy (Gorio *et al.*, 1989; Smyth *et al.*, 1993). As a result of lymphocyte depletion, especially of CD8+

T cells and impairment of cytokine production, immunosuppression and increased susceptibility to various viral and bacterial pathogens may occur (Adair, 2000; Todd, 2000; Ragland *et al.*, 2002). More complicated and severe diseases develop after co-infection with Marek's disease virus (Jeurissen & de Boer, 1993; Miles *et al.*, 2001), or infectious bursal disease virus (IBDV) (Yuasa *et al.*, 1980a, Cloud *et al.*, 1992a,b; Imai *et al.*, 1999; Toro *et al.*, 2009). Evaluation of the role, which CAV plays in the etiology of some multifactorial diseases and pathological conditions in fowl is complicated, since clinical signs, induced by the concurrent infections may prevail. Thus, the determination of the impact of the virus in any particular case requires a complex approach and application of wide spectrum of diagnostic assays. CAV has been identified using different virological and serological methods and molecular techniques such as virus isolation in cell cultures and SPF chickens, immunocytochemistry and PCR (Schat, 2003). Although at present the virus isolation was displaced by other detection methods, predominantly by DNA-based molecular techniques, historically it is one of the first methods for virus detection and identification. Irrespective of being slow and laborious, the isolation of CAV in cell cultures is indicative for the presence of infectious (not latent) virus in tested sample and enables its further antigenic and molecular characterisation. Thus, any attempts in this direction could contribute to the more detailed elucidation of the biological characteristics of circulating CAV strains.

In this paper, we describe the isolation in cell culture and identification by PCR of a field isolate of CAV from clinically ill chickens, in which co-infection with another viral agent, suggestive for IBDV

was also detected. This is the first isolation of CAV in Bulgaria.

MATERIALS AND METHODS

Case history

Seven nine-week-old egg-laying-type chickens were submitted to the laboratory for routine diagnostic investigation. Chickens originated from a flock on a small farm, comprising around 2,000 birds, raised from one-day old age to 5–6 months when they are marketed as pullets. Within two weeks the flock has experienced a disease, characterised by sudden onset of depression, prostration and increased mortality rate reaching 7.5%. The birds had been vaccinated on the first day post hatch against Marek's disease virus, but not against IBDV or other viruses. No information was available for the vaccination history of the breeder flock from which these chickens originated.

Laboratory investigations

Gross pathological and microbiological investigations were performed at necropsy. For virus isolation, tissue samples from thymuses, bursas of Fabricius (BF), livers and spleens were collected and homogenised in sterile PBS, pH 7.5, containing antibiotics. The homogenates were centrifuged at 3,000 rpm for 15 min and supernatants filtered through 0.45 µm syringe filter. Samples from the above mentioned organs including also bone marrow, were fixed in 10% neutral formalin, paraffin embedded and processed for routine histopathological investigation. The sections were stained with haematoxylin and eosin and observed under light microscope Leika DM5000B, equipped with Leika DFC420 camera. A pool of homogenised organ samples was tested for

presence of Newcastle disease virus (NDV) by rRT-PCR. To detect the presence of pathogenic bacteria, routine bacteriological procedures, including sampling of lung, liver, ileo-caecal junction and bone marrow specimens followed by inoculation onto BPLS-agar and blood agar were performed. In addition, due to the signs of anaemia and thymus atrophy, observed in some of the surveyed chickens, tissue specimens from the thymuses of three birds were pooled for DNA extraction and investigation by PCR for CAV.

Inoculation of cell cultures and chicken embryos

Freshly seeded at a concentration of 5×10^4 /mL MDCC MSB1 cells, growing in 25 cm² tissue culture flasks (Orange Scientific) were inoculated with 500 µL of filtered organ homogenates. The cells were maintained with RPMI 1640 medium supplied with 10% foetal calf serum and antibiotics, and incubated at 38 °C in a CO₂ incubator. The infected cells were subcultured in a fresh medium every 2–3 days at a ratio 1:3 to 1:4. To test for the presence of concomitant viral agents, organ homogenates were inoculated also in the yolk sac of five 8-day-old chicken embryos (CEs) as well as in cultures of chicken embryo fibroblast cells (CEF). These were maintained at 37 °C and three consecutive passages were performed. Non-infected cells, both MSB1 and CEF, processed as described above served as negative controls.

DNA extraction

DNA was extracted from the thymuses of three birds and from MDCC-MSB1 cells, inoculated with the fifth passage of newly isolated virus using Tissue & Cell Genomic DNA Mini kit (Guangzhou Gene-

shun Biotech., Ltd.) according to the manufacturer's instructions.

PCR analysis

The DNA of CAV was amplified using one set of primers (LKB Vertriebs GmbH) with sequences: S.1.1. 5'-AATGAACGC TCTCCAAGAAG-3' and: S.1.2. 5'-AGC GGATAGTCATAGTAGAT-3', respectively (Tham & Stanislawek, 1992), that comprised a fragment of 583 bp (positions 485–1067) of the published genome of the reference strain Cuxhaven-1. The working concentration of each primer was 10 pmol/µL. Amplifications were performed in 50 µL volume (2 µL of target DNA, 2 µL of each primer, 25 µL 2X PCR Taq Mix (Guangzhou Geneshun Biotech., Ltd) and 19 µL nuclease-free water) in an automated thermocycler (QB-96, LKB). The reactions consisted of initial denaturation step (2 min at 95 °C) followed by 30 cycles of denaturation (1 min at 95 °C), primer annealing (2 min at 56 °C), and extension (2 min at 74 °C). The final extension step was at 74 °C for 10 min. The resulting amplicons were visualised by electrophoresis in 1% agarose gel. DNA extracted from thymuses of chickens infected in another study with the Del Rose strain of CAV (Rosenberger & Cloud, 1989b), served as a positive control.

RESULTS

The birds presented were in poor body condition and some of them showed weak paleness of the carcasses. In most birds, subcutaneous and muscular haemorrhages were seen. At necropsy thymus atrophy was detected in four out of the seven chickens. BF's were pale and atrophic without haemorrhages. No visible gross pathological lesions in livers, lungs, spleens, gizzards and intestines were no-

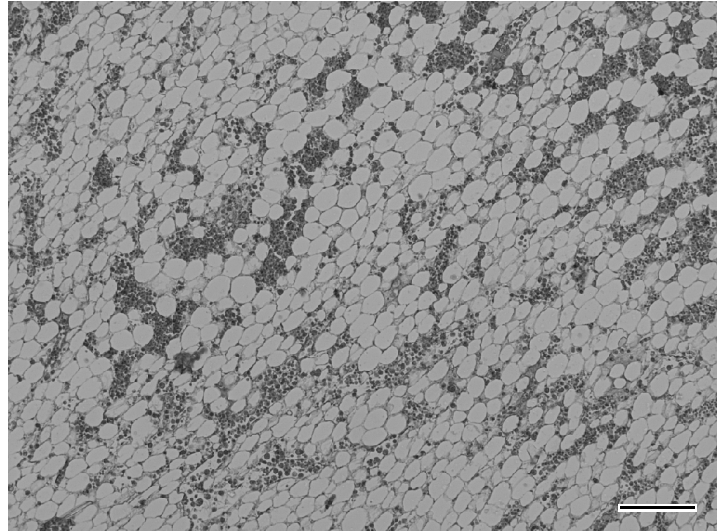


Fig. 1. Photomicrograph of bone marrow: aplastic anaemia and destruction of the haemocyto blasts, which have been replaced by adipose tissue (bar=100 μ m).

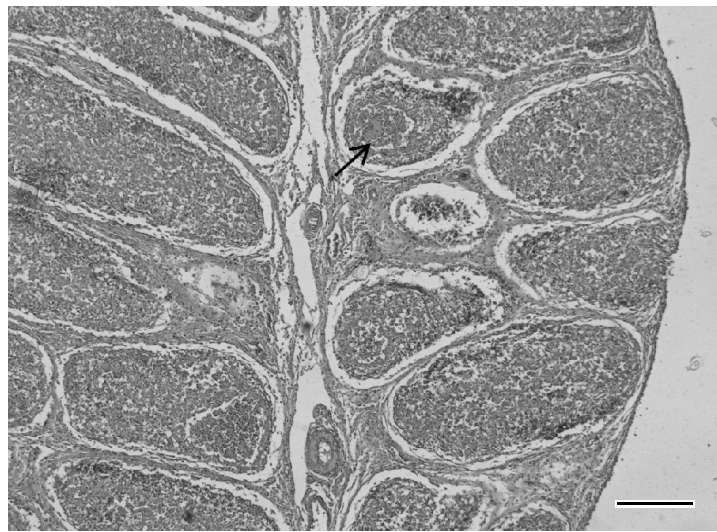


Fig. 2. Histopathological picture of chicken bursa: severe lymphocytic depletion and follicular degeneration with necroses (arrow), accompanied by oedema and widening of interfollicular connective tissue. (bar=200 μ m).

ticed. The histological observation revealed moderate lymphocytic depletion in the thymus and aplastic bone marrow (Fig. 1). Atrophy of the bursal follicles and wide-

ning of the interfollicular spaces were regular findings in most of the birds investigated (Fig. 2).

CAV PCR performed with DNA extracted from the pooled thymuses of three birds resulted in an amplification of a product with a predictable size of 583 bp (Fig. 3).

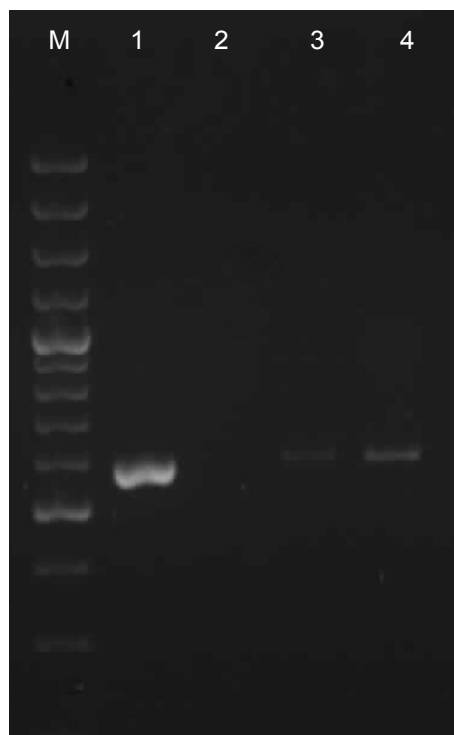


Fig. 3. Results of PCR analysis of thymus sample and infected MDCC-MSB1 cells: Lane M: 100 bp DNA marker; lane 1: Del Rose infected MSB-1 cells; lane 2: non-infected MSB-1 cells; lane 3: positive thymus sample, lane 4: CAVBG-1 infected MSB-1 cells.

After inoculation of organ homogenates in MSB1 cell cultures, signs of cytopathic effect (CPE), consisting of appearance of swollen enlarged cells, slowdown of the growth rate and cell lysis were detected still on the third passage in the inoculated cells (Fig. 4). At the same time, non-infected control cells remained unaffected. Five consecutive passages were

performed to stably adapt the isolate for growing in MSB1 cells. PCR, performed with genomic DNA, extracted from MSB1 cells that were inoculated with the fifth passage of the cytopathic agent, confirmed that it belonged to the CAV family. The putative CAV isolate was designated CAVBG-1, titrated and virus stock culture solutions were created and stored at -70°C .

Of the 5 CEs inoculated with pooled tissues homogenates, one died on the 72nd hour post inoculation (p.i.) and three died on the 120th hour p.i. All embryos showed congestion and petechial haemorrhages. On the second passage in CEs, performed with filtered ($0.45\ \mu\text{m}$) embryo homogenates and allantoic fluid from the first embryo passage, two out of five inoculated CEs died from the 3rd to the 5th days p.i. However, on the third passage none of inoculated embryos died until they were chilled and examined on the 15th day of incubation. No visible gross pathological changes were observed in these embryos except a small haemorrhage on the cerebral region of one of CEs.

No cytopathic changes were observed in the CEF cultures inoculated with organ's homogenate within three consecutive passages. Trials of pathogenic bacteria isolation as well as rRT-PCR for NDV gave negative results.

DISCUSSION

The isolation of CAV in cell cultures is a laborious process requiring sometimes up to 10 passages until CPE appeared (Mc Nulthy, 1998), and isolates that lack the ability to infect and propagate on routinely used MSB1 cell line exist (Renshaw *et al.*, 1996; Calnek *et al.*, 2000; Nogueira *et al.*, 2007). Nevertheless, in this study we could isolate a strain of CAV and ob-

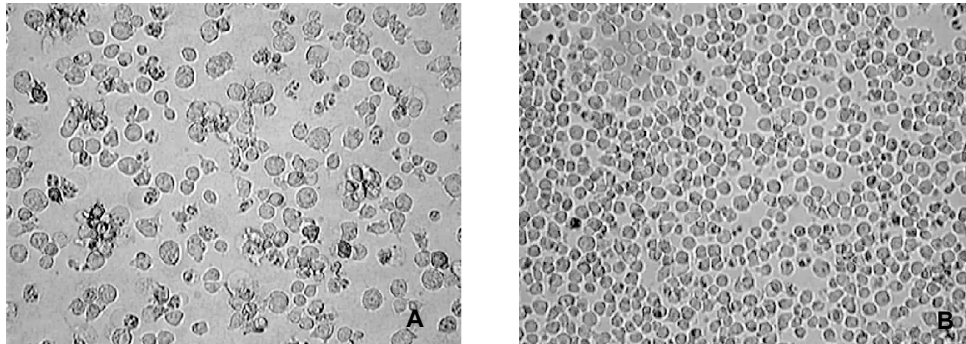


Fig. 4. Cytopathology of CAVBG-1 in MSB1 cells, 48 h post inoculation. **A.** Enlargement and granulation of cells and slowdown of cell growth in inoculated cell; **B.** Non-infected MSB1 control cells.

served appearance of CPE even on the third passage in MSB1 cells. The changes induced by CAVBG-1 in MSB1 cells were identical to the CPE described for other CAV strains. At the same time, no CPE was induced when organ homogenates were inoculated in CEF cells. This is in agreement with other reports, showing strong affinity of CAV for lymphoid tissue and the inability of the virus to replicate in conventional monolayer cell cultures (Haridy *et al.*, 2010). The positive result from the PCR, performed with DNA extracted from inoculated MSB1 cells definitively confirmed the belonging of the new isolate to the CAV and substantiated its successful adaptation for growing *in vitro*. This is the first isolation of a field strain of CAV in Bulgaria.

CAV causes clinical disease in transovarially infected chickens not protected by maternal antibodies in the first 2 to 3 weeks of life (Yuasa *et al.*, 1980b). However, due to the high immune background of the breeder flocks the cases of clinically expressed illness are rare. The birds from which we could isolate CAV were nine-week-old, suggesting that they have been horizontally infected after the depletion of the maternal antibodies. Although environmentally acquired CAV infections

in fully immunocompetent chickens are usually subclinical after 2 weeks of age, this resistance could be overcome as a result of immunosuppression, mostly due to infections caused by immunosuppressive viruses. In examined birds, together with the isolation of CAV we identified also a presence of another viral agent which caused a death of inoculated CEs between p.i. days 3 and 5. Based on the gross and histopathological findings in the examined birds (prominent bursal atrophy and subcutaneous and muscular haemorrhages) and the pathological lesions in the infected embryos as well, compatible with those described in cases of IBDV infection (Lukert & Saif, 2003), it was suspected as IBDV. Although some intermediate plus IBD vaccine strains have a potential to induce mild to moderate immunosuppression which may facilitate the replication of existing CAV infection (Chacón *et al.* 2010), the chickens studied herein have not been vaccinated against IBDV, hence probably they have experienced an infection with a wild-type IBDV. We suppose that in this case a previous infection with IBDV has promoted sufficient replication of CAV, which additionally has aggravated the clinical picture. This is in agreement with the results

from clinical cases and experimental data, showing synergistic action of both infections (Otaki *et al.*, 1989).

It is known that CAV may persist in some organs (gonads and spleens) for a long time without clinical manifestations (Brentano *et al.*, 2005). The positive results from PCR in these cases could reflect detection of a latent virus. However, in this study, the isolation of CAV in cell cultures from target organs indicates an active virus replication. Moreover, the early development of CPE in inoculated cell cultures (as early on the 3rd passage on MSB1) suggests for a presence of high viral load. Expressed histopathological lesions, including depletion of the lymphoid cells in the thymus and destruction of the hemocytoblasts in the bone marrow, indicate an active infection. Taken together, these facts definitively show that CAV has played an important role in the etiopathogenesis of this clinical case.

The identification of CAV in diseased 2-month-old chickens which are thought not to be at risk with regard to clinical infectious anaemia shows, that even at this age the virus can cause problems in the field. This requires consideration of CAV in the diagnostic scheme of such birds too.

REFERENCES

- Adair, B. M., 2000. Immunopathogenesis of chicken anemia virus infection. *Developmental and Comparative Immunology*, **24**, 247–255.
- Brentano, A., S. Lazzarina, S. S. Bassia, T. A. P. Kleina & K. A. Schat, 2005. Detection of chicken anemia virus in the gonads and in the progeny of broiler breeder hens with high neutralizing antibody titers. *Veterinary Microbiology*, **105**, 65–72.
- 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.
- Chacón, J., E. Nogueira, L. Brentano, C. Gomes, C. Astolfi-Ferreira, L. Villarreal & A. Ferreira, 2010. Detection of chicken anemia virus and infectious bursal disease virus co-infection in broilers. *Brazilian Journal of Veterinary Research and Animal Science*, **47**, 293–297.
- Cloud, S. S., H. S. Lillehoj & J. K. Rosenberger, 1992a. Immune dysfunction following infection with chicken anemia agent and infectious bursal disease virus. 1. Kinetic alterations of avian lymphocyte subpopulations. *Veterinary Immunology and Immunopathology*, **34**, 337–352.
- Cloud, S. S., J. K. Rosenberger & H. S. Lillehoj, 1992b. Immune dysfunction following infection with chicken anemia agent and infectious bursal disease virus. 2. Alterations of *in vitro* lymphoproliferation and *in vivo* immune responses. *Veterinary Immunology and Immunopathology*, **34**, 353–366.
- Gorio, M., T. Suwa, T. Umemura, C. Utakura & S. Yamashiro, 1989. Histopathology of chicks inoculated with chicken anaemia agent (MSB1-TK5803 strain). *Avian Pathology*, **18**, 73–89.
- Haridy, M., M. Goryo, J. Sasaki & K. Okada, 2010. Comparative susceptibility of some avian and mammalian lymphoblastoid cell lines to chicken anemia virus. *Journal of Veterinary Medical Science*, **72**, 1637–1641.
- Imai, K., M. Mase, K. Tsukamoto, H. Hihara & N. Yuasa, 1999. Persistent infection with chicken anaemia virus and some effects of highly virulent infectious bursal disease virus infection on its persistency. *Research in Veterinary Science*, **67**, 233–238.
- Jeurissen, S. H. M. & G. F. de Boer, 1993. Chicken anaemia virus influences the pathogenesis of Marek's disease in experimental infections, depending on the dose of Marek's disease virus. *Veterinary Quarterly*, **15**, 81–84.

- Lukert, P. & Y. Saif, 2003. Infectious bursal disease. In: *Diseases of Poultry*. 11th edn, eds Saif, Y. M., H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne, Iowa State University, Ames, pp. 161–180.
- Mc Nulty, M. S., 1998. Chicken anemia virus. In: *A Laboratory Manual for the Isolation and Identification of Avian Pathogens*, 4th edn, AAAP, University of Pennsylvania, New Bolton Center, Kennett Square, PA, USA, pp. 146–149.
- Miles, A., S. Reddy & R. 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.
- Nogueira, E. O., A. J. P. Ferreira, R. M. Soares, E. L. Durigon, S. Lazzarin & L. Brentano, 2007. Genome sequencing analysis of Brazilian chicken anemia virus isolates that lack MSB1 cell tropism. *Comparative Immunology, Microbiology and Infectious Diseases*, **30**, 81–96.
- Otake, Y., T. Nunoya, M. Tajima, K. Saito & Y. Nomura, 1989. Enhanced pathogenicity of chicken anemia agent by infectious bursal disease virus relating to the occurrence of Marek's disease vaccination breaks. *Japanese Journal of Veterinary Sciences*, **51**, 849–852.
- Pringle, C. R., 1999. Virus Taxonomy at the XIth International Congress of Virology, Sydney, Australia. *Archives of Virology*, **144**, 2065–2070.
- Ragland, W. L., R. Novak, J. El-Attrache, V. Savič & K. Ester, 2002. Chicken anemia virus and infectious bursal disease virus interfere with transcription of chicken IFN-alpha and IFN-gamma mRNA. *Journal of Interferon and Cytokine Research*, **22**, 437–441.
- Renshaw, R. W., C. Soine, T. Weinkle, P. H. O'Connell, K. Ohashi, S. Watson, B. Lucio, S. Harrington & K. A. Schat, 1996. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. *Journal of Virology*, **70**, 8872–8878.
- Rosenberger, J. K. & S. S. Cloud, 1989a. The effects of age, route of exposure, and coinfection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). *Avian Diseases*, **33**, 753–759.
- Rosenberger, J. K. & S. S. Cloud, 1989b. The isolation and characterization of chicken anemia agent (CAA) from broilers in the United States. *Avian Diseases*, **33**, 707–713.
- Schat, K. A., 2003. Chicken infectious anemia. In: *Diseases of Poultry*. 11th edn, eds Saif, Y. M., H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne, Iowa State University, Ames, pp. 182–202.
- Smyth, J., D. Moffett, M. McNulty, D. Todd & D. Mackie, 1993. A sequential histopathologic and immunocytochemical study of chicken anemia virus infection at one day of age. *Avian Diseases*, **37**, 324–338.
- Tham, K. M. & W. L. Stanislawek, 1992. Polymerase chain reaction amplification for direct detection of chicken anemia virus DNA in tissues and sera. *Avian Diseases*, **36**, 1000–1006.
- Todd, D., 2000. Circoviruses: Immunosuppressive threat to avian species; a review. *Avian Pathology*, **29**, 373–394.
- 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.
- Toro, H., V. L. van Santen, F. J. Hoerr & C. Breedlove, 2009. Effects of chicken anemia virus and infectious bursal disease virus in commercial chickens. *Avian Diseases*, **53**, 94–102.
- Yuasa, N., T. Taniguchi, T. Noguchi, & I. Yoshida, 1980a. Effect of infectious bursal disease virus infection on incidence of anemia by chicken anemia agent. *Avian Diseases*, **24**, 202–209.
- Yuasa, N., T. Noguchi, K. Furuta & I. Yoshida, 1980b. Maternal antibody and its ef-

Isolation and PCR identification of chicken anaemia virus infection in Bulgaria

fect on the susceptibility of chicks to chicken anemia agent. *Avian Diseases*, **24**, 197–201.

Correspondence:

Dr. Konstantin Simeonov
Department of Virology
National Diagnostic & Research Veterinary
Medical Institute
15, Pencho Slaveikov Bld.
1606, Sofia, Bulgaria
email: kbsimeonov@yahoo.com

Paper received 17.10.2013; accepted for
publication 20.02.2014