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Chicken infectious anaemia virus infection among broiler chicken flocks in Iran

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ABSTRACT

Chicken infectious anaemia is a viral disease in young chickens which characterized by aplastic anaemia and immunosuppression. Between January 2004 and July 2005, an unusual hemorrhage in subcutaneous and intramuscular tissues of broiler chickens at slaughter houses of Mashhad, Isfahan and Tehran provinces were occurred. Postmortem examination revealed severe hemorrhages in the wings and muscles of the legs and atrophy of the thymus in all the affected chicks. Twenty two flocks, collected from slaughter houses of these provinces investigated in this study. PCR was carried out for detection of DNA virus in pooled liver and thymus suspensions and blood samples were collected for ELISA assay. All of the collected tissue samples from the affected flocks were found to be positive. Totally 440 serum samples collected from the affected flocks were tested in which 316 (71.8%) of the sera were seropositive with seroprevalence ranging from 25% to 100%. The number of PCR positive samples was significantly higher than seropositive samples measured by ELISA. In conclusion, it seems that CIAV has a widespread distribution among the Iranian broiler flocks and the virus plays a critical role in development of hemorrhage in broiler chickens at the slaughter houses.

Keywords: Chicken infectious anaemia virus (CIAV), PCR, ELISA, Iran

INTRODUCTION

Chicken infectious anaemia virus (CIAV) was first described as a Circovirus in 1979 by Yuasa in Japan and has since been observed in many other countries throughout the world. The CIAV is a very small (25 nm) ssDNA circular molecule of about 2300 nucleotides with three open reading frames (McNulty *et al* 1990, Yuasa *et al* 1979). The virus is non-enveloped, ubiquitous and highly resistant to thermal inactivation and treatment with lipid solvents and many of the common disinfectants (John 1998). In the *Circoviridae*, viruses of porcine circovirus (PCV) and beak and feather disease virus (PBFDV) are classified in the genus Circovirus, while chicken anaemia virus is the only member of the genus Gyrovirus (Crowther *et al* 2003). The virus genome encodes one structural (VP1) and two nonstructural (VP2 and VP3) proteins (Todd *et al* 2002). The virus can be detected in affected chickens by virus isolation, immunohistochemistry and immuno-fluorescence techniques and a variety of

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molecular methods. ELISA assay is the preferred method for detecting antibodies to CIAV.

CIA causes bone marrow aplasia with severe anaemia, thymus, bursa, spleen atrophy and hemorrhages of the proventriculus and skeletal muscles and mortality in 2-3-weeks olds chicks (Yuasa *et al* 1979, Taniguchi *et al* 1982). In young chicks, the infection may display different signs and various degrees of severity. The disease is usually complicated by secondary viral, bacterial or fungal Infections (Vegad 2001). It has also been reported a loss of net income due to decreased weight at processing and increased mortality around 3 weeks of age in broiler flocks (McIlroy *et al* 1992). The subclinical infections of commercially produced broilers might result in increased mortality and condemnations (McNulty *et al* 1988). Although the maternal immunity transferred to progeny can prevent clinical manifestation of chicken infectious anaemia, subclinical infection may occur and be synergized by diseases caused by other pathogens especially immunosuppressive viruses (Rosenberger 1998). Diagnosis of CIAV infections can be made by detecting infectious virus, viral antigens, viral DNA or virus specific antibodies (Chettle *et al* 1989). The first occurrence of CIA infection in broiler chicken flocks of Iran has been reported by Toroghi *et al* in 2003. Then, several clinical cases and numerous subclinical cases of CIAV infection were also recorded by Bassami (Unpublished data). Due to the occurrence of severe hemorrhage in subcutaneous and intramuscular tissues of broiler chickens at slaughter houses of Mashhad, Isfahan and Tehran provinces, this study was conducted to demonstrate the presence of CIAV infection in three major provinces in Iran.

MATERIALS AND METHODS

The flocks. The tissue samples and blood from twenty two affected flocks at 8–10 weeks old from the slaughter houses of Mashhad, Isfahan and Tehran provinces were collected. The most consistent finding in all of the affected carcasses included subcutaneous and intramuscular hemorrhages, atrophy of thymus and undersized weight. Five broiler flocks from the slaughter houses of Tehran province with no apparent hemorrhage manifestation were defined as negative control samples. 440 blood samples (20 samples from each flock) were collected for ELISA assay. Samples were collected from liver and thymus of each flock were pooled, homogenized and stored at -70 °C for DNA extraction. The information about the flocks is summarized in Table 1.

DNA extraction. The tissue samples of thymus and liver from each flock were homogenized, followed by DNA extraction using High Pure Viral Nucleic Acid Kit (Roche, Germany), according to manufacturer's instruction.

Polymerase chain reaction (PCR). PCR was performed to amplify a fragment of 454 bp from the VP1 (capsid) gene of CAV. The oligonucleotide primers were designed based on the published DNA sequence of Cux-1 strain of CIAV. The sequences of the primer are as follows:

Formard primer: (5' – AGC CGA CCC CGA ACC GCA AGAA – 3')

Reverse primer: (5' – AGA CCC GTC CGC AAT CAA CTC ACC – 3')

The PCR mixture consisted of 1.5 mM $MgCl_2$, 200 µM of the dNTPs, 2.5 unit Thermus aquaticus DNA polymerase enzyme, 15 pM of each primer, and 2 µl of template DNA in a total volume of 50 µl. Following an initial 2.5 minutes denaturation step at 94 °C, 30 thermal cycles, with each cycle comprising 1 minute at 94 °C, 30 seconds at 55 °C and 1 minute. at 72 °C, were carried out. A final extension period of 7 minutes at 72 °C preceded storage of the reaction products at 4 °C. All DNA amplifications were performed in an automatic Ependorf thermal cycler. The PCR products were analyzed by electrophoresis in 1% agarose gels, stained with

ethidium bromide and visualized by using an ultraviolet transilluminator.

Table 1. Flocks and collected samples data.

Parameters	Mashhad	Tehran	Isfahan
Flock size (total)	40,000	120,000	40,000
Flocks number	5	17	5
Slaughter age (wks)	8-10	8-10	8-10
Number of tissue samples collected per flock (for PCR test)	20	20	20
Number of blood samples per flock (for ELISA assay)	20	20	20
Extent of hemorrhage in affected flocks	++	+	+++

(+) Mild hemorrhage, (++) Moderate hemorrhage, (+++) severe hemorrhage.

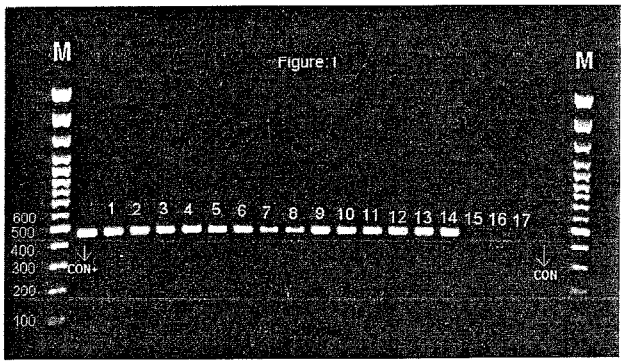

Figure 1. PCR detection of CAV in the pooled tissues (liver and spleen). A sample of Agarose gel electrophoresis of PCR products using a specific primer for CAV. *Lanes* 1-12 (some of the affected flocks), *Lanes* 13-17 (negative control flocks), CON+ (control positive), CON- (control negative) and M (100bp DNA marker).

ELISA. In total 440 sera collected from the flocks (20 samples for each flock) were tested by a commercial ELISA test kit, according to the instructions by the manufacturer (FlockChek, CIAV, IDEXX). Optical density values were read at 650 nm using a Multiscan ELISA reader (Titertek). According to the manufacturer, an optical density of equal or greater than 0.60 was defined as a negative sample and a density of equal or less than 0.5 was interpreted as a positive sample.

RESULTS

PCR. The PCR results are shown graphically in Figure 1. Ethidium bromide-agarose gel electrophoresis revealed a specific amplified fragment of 454bp. All 22 pooled samples of thymus and liver homogenates in the affected flocks were positive by PCR (%100) whereas two of five control flocks found to be positive.

ELISA. In Table 2 shows the result of ELISA assay. 316 (%71.8) out of 440 collected sera samples from the affected flocks were positive in ELISA assay while only 41 sera (41%) were positive among the control flocks. One flock in the latter group was found to be free of CIA infection.

DISCUSSION

The clinical disease of chicken infectious anaemia virus (CIAV) is rare today because of the widespread practice of vaccinating breeders, but the subclinical form of the disease is ubiquitous (Sommer and Cardona 2003). Several studies have confirmed the harmful effects of the clinical and subclinical infections by chicken infectious anaemia virus (CIAV) (Hagood *et al* 2000, McIlroy *et al* 1992, McNulty *et al* 1990, McNulty *et al* 1991).

However, the final effect is the expression of multiple roots such as management, stress, genetic background and additional pathogens. The virus is a major cause of cell-mediated immunosuppression. Schat (2004) has shown an obvious decline in the health, productivity and profitability in the affected flocks. Between January 2004 and July 2005 the occurrence of severe hemorrhage in subcutaneous and intramuscular tissues of broiler chickens after slaughtering processes were investigated for CIAV infection. The results of genome detection and serological profile of the affected flocks clearly enough demonstrated the presence of infection in a great extent. The PCR assay showed that 100% of samples collected at some slaughter houses of the three provinces were positive for CIAV.

Table 2. Comparison of the PCR and ELISA assays in the affected and control flocks.

No	Farm	Number of	PCR
1	T1	19/20	+
2	T2	10/20	+
3	T3	11/20	+
4	T4	15/20	+
5	T5	19/20	+
6	T6	14/20	+
7	T7	12/20	+
8	T8	14/20	+
9	T9	5/20	+
10	T10	14/20	+
11	T11	18/20	+
12	T12	11/20	+
13	T13	14/20	+
14	T14	18/20	+
15	T15	0/20	-
16	T16	4/20	-
17	T17	5/20	-
18	M1	20/20	+
19	M2	17/20	+
20	M3	14/20	+
21	M4	14/20	+
22	M5	20/20	+
23	E1	9/20	+
24	E2	18/20	+
25	E3	14/20	+
26	E4	13/20	+
27	E5	15/20	+

Abbreviations: T: *Tehran*, M: *Mashhad*, E: *Isfahan* and T13-T17: *Negative control flocks.*

Two of five apparently healthy flocks, sampled as negative flocks, were also turned-up to be positive in PCR assay. This finding is not surprising, as the presence of DNA of CIAV is so widespread that SPF flocks have no way to escape the infection. The ELISA assay also confirmed the dynamics of infections in the studied flocks. The results of this study strongly indicate the widespread distribution of infections among Iranian poultry farms.

The interesting finding in our study was the extent of hemorrhage in subcutaneous and intramuscular tissues after slaughtering, as this feature is more prominent in clinical cases of CIAV infection. In comparison to control flocks, it seems that CIAV plays a critical role in development of hemorrhage on carcasses at slaughter houses. However, the contribution of other causes such as Marek's disease virus and/or infectious bursal disease should not be ignored. Miles *et al* (2001) found that co-infection with CIAV and very virulent (vv) MDV strains exacerbated the mortality and thymus atrophy. The new findings have been showed that CIAV is capable of infecting thymocytes of older birds, in contrast to previous belief, which is associated with lymphocyte depletion (Smyth *et al* 2006). The age of flocks investigated ranged from 8 to 10 weeks. The strains of broiler chickens reared in Iran are among the top commercial strains produced in developed countries and the performance of theses strains in Iranian broiler flocks is, some how comparable to standards of these strains. Based on these facts, the age of flocks slaughtered at 8 to 10 weeks, clearly indicate the low performance of infected flocks investigated. Unfortunately, the information about the production performances of the flocks was not accessible, therefore the only indication for low performance of the affected flocks was the age at slaughterhouse and the sub-standard sizes of the chicken slaughtered.

In comparison to very high percentage (100%) of PCR positive samples, the numbers of serologically positive chickens in the affected flocks were 71.8%. Due to lack of vaccination practice against CIAV among Iranian broiler breeders and based on the uneven levels of maternal antibodies, the clearance of these antibodies occurs in different ages. Moreover it has been repotted that some strains of CIA are able to cause clinical disease after experimental infection of 10 weeks old broiler breeders (Toro 1997). However, due to horizontal transmission of CIAV, the individual chicks will be consequently infected at various ages and gradually develop antibody against the virus. It is very clear that the presence of serologically positive chickens at 8 to 10 weeks old result from CIAV infection. These facts could clearly explain the differences in the number of seropositive individuals among infected flocks and also explain why most of the chickens are

expected to be positive by PCR. Therefore, in infected individuals, the genome of CIAV can easily be picked-up by PCR, in which millions copy of target DNA are amplified. Based on above explanation it might be reasonable to expect that if the broiler chickens are not slaughtered at ordinary age, they will be seropositive later on their life. Studies on dynamics of infection in two commercial broiler conducted by Sommer & Cardona (2003) is totally in harmony with our results. They found that after natural challenge of the flocks by field virus, 80% of the flock were positive by PCR assay, but only 35% of the same birds were positive by ELISA assay. One week later 90% of flocks were positive by PCR and ELISA assays. The result obtained by Owoade *et al* confirms this speculation. They found that 86% of younger broiler flocks and 100% of flocks older than 6 to 8 weeks old were positive by ELISA. Brentano *et al* found that 89% of sera tested by a commercial ELISA assay were positive for antibodies to CAV in Brazil. Due to widespread distribution of infection and dynamics of infection it could be speculated if the sampling is optimized many negative samples might turn up to be positive.

In conclusion, these studies will contribute to a better understanding of widespread distribution of CIAV infection in Iranian poultry industries and shedding a light in possible great role of this infection in immunosuppression with all consequences. This study also demonstrates the high level of horizontal transmission and confirms the necessity of an even level of maternal antibodies against CIAV infection. Due to immunosuppression caused by subclinical infection it may also be a good indicator for needs for the development of an attenuated vaccine to be safely used in day-old chicks, without interfering with maternal antibodies. To have a better understanding about what is happening in Iranian poultry industry it is essential that we determine the distribution of CIA in large population of chicken in Iran and analyze its role in the aggravation of other infectious diseases.

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عفونت ویروس کم خونی عفونی جوجه‌هادر گله‌های گوشتی ایران

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خلاصه

کم خونی عفونی جوجه‌ها یک بیماری ویروسی است که در جوجه‌های جوان با کم خونی آپلاستیک و تضعیف سیستم ایمنی مشخص می‌گردد. در فاصله زمانی، زمستان ۸۴ تا تابستان ۸۵ خونریزی‌های غیرمعمول در بافت‌های زیر جلد و عضلات جوجه‌های گوشتی در کشتارگاههای استان خراسان، اصفهان و تهران به وقوع پیوست. در معاینات گالبدگشایی خونریزی‌های شدید در بال‌ها، عضلات پا و آتروفی تیموس در تمام جوجه‌های مبتلا آشکار شد. بیست و دو گله مرغ گوشتی از کشتارگاههای این استانها تحت مطالعه قرار گرفتند، آزمایش PCR جهت تعیین DNA ویروس از مخلوط بافت‌های صلایه شده کبد و تیموس انجام شد و همچنین نمونه‌های خون جهت بررسی توسط آزمایش الایزا برداشت شد. تمامی نمونه بافت‌های جمع آوری شده از گله های مبتلا در آزمایشPCR مثبت ارزیابی شدند، از ۴۴۰ نمونه سرم جمع‌آوری شده از گله های مبتلا ۳۱۶ نمونه (۷۱/۸درصد) با دامنه حداقل ۲۵ درصد تا ۱۰۰ درصد مثبت بودند. تعداد نمونه‌های مثبت در آزمایش PCR بصورت معنی داری از نمونه‌های سرم مثبت در آزمایش الایزا بیشتر بود. در نتیجه به نظر می‌رسد که ویروس کم خونی جوجه ها در بین گله‌های مرغ گوشتی ایران گستردگی وسیعی داشته و این ویروس نقش عمده ای در ایجاد خونریزیهای کشتارگاهی مرغ های گوشتی ایفا می نماید.

واژه های کلیدی: ویروس کم خونی عفونی جوجه‌ها، PCR، ELISA ، ایران