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CHICKEN INFECTIOUS ANEMIA VIRUS: VIRUS ISOLATION, SEROTYPING AND SEROLOGIC DIAGNOSIS OF NATURAL INFECTION IN BROILER CHICKENS FLOCKS

(With 3 Tables and One Figure)

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تشخيص العدوى الطبيعية لفيروس فقر الدم المعدى فى الدواجن بالعزل والتشخيص السيرولوجى بين قطعان دجاج اللحم

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يتميز فيروس فقر الدم المعدى بالعدوى واسعة الأنتشار بين قطعان الدواجن. وجهت هذه الدراسة للتشخيص الأولى للعدوى الطبيعية بالفيروس باستخدام العزل بالحقن فى بيض الدجاج المخصب والكتاكيت عمر يومين بالاضافة إلى التشخيص السيرولوجى. تم تجميع عينات نسيجية من الأعضاء الداخلية للطيور المصابة من ٢٢ قطيع من الدجاج اللاحم عمر من ١-٦ أسابيع. ظهر هناك التهاب عام ونزف منتشر على أجنة البيض المحقون وخاصة بعد التمريرة الثالثة بينما حصاد البيض المحقون لم يعطى نتائج إيجابية مع اختبار التلازن الدموى وكذلك أختبار الترسيب باستخدام الأجسام المضادة المرجعية. بالنسبة للحقن فى الكتاكيت فقد لوحظ وجود وفيات بمعدل ٤،٥% وآثار مرضية متوسطة فى ٩ مجاميع من اجمالى ٢٢ عينة (٤١%) بالاضافة إلى قياس الأجسام المضادة الخاصة بالفيروس فى الدم باستخدام الاليزا والتى أعطت نسب ٣٢%، ٤٥% عند اليوم ٢١،١٤ بعد الحقن وبمعدلات عيارية بلغت ٩٠٧،٢٧٦ على التوالى. تم قياس الأجسام المضادة الخاصة بالفيروس فى ٤٦٧ عينة مصل من هذه القطعان بعدد ٨-٣٤ عينة من كل قطيع/ مزرعة باستخدام أختبار الاليزا. إن الغالبية العظمى من القطعان (٢١-٢٢) وبنسب مئوية من صفر الى ١٠٠% أظهرت ايجابية عالية للأجسام المضادة. كذلك أظهرت النتائج أن هناك ٣٧٥ عينة من ٤٦٧ (٨٠%) موجبة بمعدلات عيارية للأجسام المضادة بلغت ٧٢١٥. هذه الدراسة هي الأولى لتشخيص فيروس فقر الدم المعدى فى الدواجن بين قطعان دجاج اللحم والتي أظهرت أن هناك توافق بين العزل وبين شكوي أصحاب المزارع من معدلات قلة الوزن والوفيات بالاضافة الى فشل التحصين ضد الفيروسات الأخري.

SUMMARY

Chicken infectious anemia virus (CIAV) is ubiquitous worldwide pathogen affecting chickens flocks. This study was directed to investigate isolation, identification and serologic prevalence of natural infection with CIAV in broiler chickens. A total of 22 different unvaccinated flocks/farms between 1 and 6 weeks of age, were sampled by pooled organ tissues (1 pool per flock) for virus isolation on SPF eggs and chick inoculation. On chicken embryos, changes of general mild edema, inflammation and few hemorrhages following to three blind serial passages were reported. The egg harvests failed to agglutinate 1% avian erythrocytes or precipitate in agarose against SPAFAS CIAV antiserum. Post chicks inoculation; low mortalities (4.5%) and mild gross lesions were observed in 9 groups of 22 by 41%. The inoculated chicks developed low to moderate titers of antibodies on days 14 and 21 by percentages of 32% and 45% with mean titers of 276 and 907 respectively. A total of 467 chicken serum samples representing the 22 broiler flocks ranged from 8-34 samples per farm were collected and tested by indirect Enzyme Linked ImmunoSorbent Assay (ELISA). Most chicken flocks 21 of 22 (95%) showed seropositivity by percentages ranged from 0% to 100% by a total number of 375/467 (80%) that was higher in older broilers than younger within the susceptible age. The log of antibody titers among the flocks ranged from 1.855 to 4.203 by titers of 72 to 15955 by mean of 7215. The presence of CIAV antibodies in the sera obtained either from field (95%) or inoculated chickens (32% to 45%) was positively correlated with the owners complains of poor vaccination responses against other infections, either viral or bacterial. However, the obtained results gave proof of widespread endemic status of CIAV exposure and infection in chickens.

Key Words: CIAV, virus isolation, egg embryos, chicks, sero-prevalence, ELISA, broilers.

INTRODUCTION

CIAV is acute, subclinical immunosuppressive worldwide pathogen affecting only chickens particularly ages of 2-4 weeks. It was first reported in Japan (Yuasa *et al.*, 1979), in USA (Rosenberger and Cloud, 1989a and 1998). Since then, it has been demonstrated in various countries; England (Chettle *et al.*, 1989), Australia (Firth and Imai,

1990), Brazil (Brentano *et al.*, 1991); South Africa (Witcht and Maharaj, 1993); China (Zhou *et al.*, 1997).

CIAV is classified within the *Genus Gyrovirus* in the *Family Circoviridae*. It is small (16-22 nm), non enveloped, icosahedral virus that contains single stranded DNA genome of 2.3 kb. The virus is stable against wide range of temperature, pH, lipid solvents and common disinfectants. Antigenically, all detected Japanese, European, and American isolates of CIAV are of the same immunological serotype (Gelderblom *et al.*, 1989). The virus can be transmitted vertically through the eggs of seronegative infected breeders (until the breeder develops antibodies) as well as with semen from viremic cocks during the breeding time. Horizontal transmission occurs by ingestion; inhalation as well as direct or indirect contact with fomites contaminated with faces of vertically infected birds. Mechanically from farm to farm, the virus can be transmitted via people, equipments and vehicles (Bulow and Schat, 1997; Hoop, 1993).

CIAV induces in vivo down regulation of cell mediated immune function through up regulation of apoptosis of thymic cortical thymocytes, decreasing of T-cell growth factor, lymphocytic transformation, interferon and interleukin-1 production, FCR expression as well as phagocytosis by macrophages. In addition, in vitro apoptosis of infected lymphoblastoid cell lines (Cloud *et al.*, 1992). Growing broilers could be vertically or horizontally infected immediately after hatching with CIAV which manifest many losses due to the serious damage of their organs by the virus. The affected chickens show poor performance, depression, severe anemia, reduced body weight, frequent dermatitis, poor vaccination responses to other infections and atrophy of both thymus and bone marrow with transient congestion of spleen and Bursa of Fabricious particularly in vertically infected chickens 10 to 14 days of age. It is accompanied with cutaneous, subcutaneous and muscle hemorrhages particularly in the wing tips (blue wing) and on the proventricular mucosa. Variable morbidities and mortalities of 45% to 60% were reported particularly among vertically infected flocks by 2-4 weeks of age. An immunosuppression either alone or in combination with other agents such as Marek's and infectious bursal viruses was developed. With age and seroconversion, survivors with sub-clinical infection usually return to normal by the 5th week of age and often stunted (McNulty, 1991).

The pathogenesis of CIAV infection is strongly related to humoral immunity, and the presence of maternal antibody completely inhibited CIAV infection. However, CIAV can be recovered from the organs of inoculated chickens for an extended period of time even after virus specific antibody was developed (Yuasa *et al*., 1980a).

Control of virus infection can be mediated by passively acquired maternal antibody, feeding of chicks with infected chicken liver homogenates and vaccination before the onset of production in order to ensure sufficient levels of protective antibodies in the offspring progeny (Fussell, 1998). In this study, neither vaccination has directed nor has report of CIAV infection in the sampled chickens. The present work was to carry out an investigation that included isolation, identification and serologic diagnosis of natural infection with CIAV in broiler chickens.

MATERIALS and METHODS

History and vaccination status of sampled chickens:

A total of 22 broiler chicken flocks between 1 to 6 weeks old were sampled. The chickens were floor reared under hygienic conditions and there was no contact between these farms. The chickens showed respiratory manifestations, anemia, growth retardation, depression, unthrifty, emaciation; frequent diarrheas, low production scores and frequent undetermined mortalities. On necropsy, there was thymus atrophy, edematous spleen, enlarged liver, inflammed of bursa of Fabricious, and pale bone marrow. None of the birds examined in this study was vaccinated against CIAV.

Collection of tissue samples:

Samples including: liver, thymus, cecal tonsils, bone marrow and spleen were aseptically collected from each bird then pooled per flock in a universal tube containing transport media of phosphate buffered saline with antibiotics, under cooled conditions within an ice box. A (20% w/v) homogenates were prepared, freezed and thawed twice, clarified by centrifugation at 3000 rpm and the supernatants were collected aseptically and used for virus isolation by inoculation into SPF eggs and chicks (Zhou *et al*., 1997).

Collection of serum samples:

A total of 467 blood samples representing the 22 chickens flocks by 8-34 sample per flock were collected for serum and used for measurement of specific antibodies to CIAV. All selected individual

chickens of each flock were bled and sampled only once while still alive and at necropsy. The blood samples were centrifuged at 3000 rpm for 10 minutes and the sera were separated, inactivated at 56^{0}C for 30 minutes and then all stored at -20^{0}C until tested for serologic survey by indirect CIAV ELISA (Table 1).

Reference positive and negative chicken CIAV antisera:

a- Positive heat inactivated CIAV antiserum was obtained from Charles River Labs, SPAFAS, Franklin Commons; North Franklin, CT, USA.

b- Positive CIAV antiserum was provided; ELISA kit, Synbiotics^R, KPL, USA.

c- Negative sera were collected from hatched SPF chicks and also was provided by the ELISA kit. All sera were stored in vials at -20^{0}C until used.

Isolation of CIAV using embryonated Specific Pathogen Free (SPF) chicken eggs:.

A total of 355 SPF chicken eggs (Jordan Bio-Industrise Center, JOVAC, Jordan) were inoculated with 0.2 ml of each sample via yolk, allantoic cavity and chorioallantoic membrane routes, at embryo ages of 5, 9 and 12 day respectively. Another SPF eggs were inoculated with normal PBS as control. The inoculated eggs were monitored up to the embryonic age of 18 days post inoculation for produced lesions and deaths if any. The samples were blindly passaged for three times, after each passage, the egg contents including yolk remnants, allantoic fluid, CAM, and embryonic tissues were harvested aseptically and pooled then used in the 2nd and 3rd passages. Eventually, the harvests of the 3rd passage were used in HA and immune diffusion tests.

Isolation of CIAV via SPF chicks:

Sample's supernatants were inoculated intramuscularly into the thigh of 2 days old SPF chicks (hatched in and housed in the laboratory) with 0.2 ml per bird by 2 chicks per each sample (total of 44 chicks), 7 chicks were kept as control. The inoculated chicks were kept separately and observed daily for 21 days for symptoms and deaths. Sequential blood sampling on days 14 and 21 post inoculation were collected for detection of CIAV antibody. Eventually, all chickens were euthanatized and the organs were retained for gross lesions (McNulty *et al.*, 1989b, Lamichhane *et al.*, 1991).

Serologic diagnosis using indirect ELISA test:

The presence of antibodies against CIAV in the chicken's collected sera either from the field or inoculated chicks was investigated by using CIAV-indirect ELISA (Synbiotics^{R}, KPL, Proflock^{R}, San Diego, CA, USA). The ELISA assay was conducted with a 1:50 dilution of each used serum according to the supplied manufacturer's instructions, Brewer *et al.*, 1994; and Todd *et al.*, 1990b). The formed antigen-antibody complex was detected by the addition of purified goat anti-chicken IgG (H+L) biotin, HRP-streptavidine conjugate (1:100). Chromogen substrate (ABTS), and then stop solutions were added and the optical density (OD) / absorbance was measured by using the (Dynatech MR 5000, Ireland) reader at 410 nm.

Statistical analysis:

The OD averages for both positive and negative control sera were calculated to give the Corrected Positive Control (CPC). Sample to positive (S/P) ratio was calculated using the format:

$$\text{S/P ratio} = \frac{\text{Sample OD/Absorbance- Mean of Negative OD/Absorbance}}{\text{Corrected Positive Control Absorbance}}$$

A sample considered positive for CIAV antibody when the calculated S/P ratio was higher than > 0.349. CIAV ELISA titers were calculated using the following equation:

Log_{10} Titer = (1.009 X Log_{10} S/P) + 3.628, Titer = Antilog of Log_{10} Titer.

RESULTS

Isolation of CIAV using SPF chicken eggs:

None of the inoculated egg embryos showed abnormal changes in the first and second passages. All mortalities and lesions reported in the 3rd passage were in the form of general mild edema, inflammation and few hemorrhages on the embryos. The harvested egg contents neither induced HA of chicken's erythrocytes nor diffusion in agarose with SPAFAS CIAV antiserum.

Isolation of CIAV using SPF chicks:

Few clinical signs similar to CIAV were reported among five inoculated chicks groups (# 3, 6, 8, 15, and 21 of 22) by a percentage of 23%, while low mortalities 2 of 44 (4.5%) were reported in the groups (# 3, 15). CIAV mild gross lesions were observed in 9 groups of 22 (41%) including the numbers (# 1, 3, 6, 8, 11, 12. 16, 17 and 21). The changes included dark areas on muscles, anemia, edematous liver and spleen, pale

bone marrow, undersized thymus and BF. CIAV antibodies were found mostly in groups where lesions also found. The antibody titers were low to moderate on day 14 (7/22) by 32%, and mean titer of 276 while on day 21, (10/22) by 45%, and mean titer of 907 (Table 2).

Serologic diagnosis using indirect ELISA test:

The intensity of color was directly proportional to the level of CIAV antibody in the sera. The OD values of the samples varied from 0.333 to 2.121 in comparison to the reference negative and positive sera of 0.196 and 1.133 respectively. Most chicken's flocks 21 of 22 (95%) showed significant overall sero-conversion to CIAV by percentages ranged from 0% to 100%. While among birds were 375/467 (80%) that was higher in older chickens than younger within the susceptible age. The log of antibody titers among the flocks ranged from 1.855 to 4.203 by individual titers of 72 to 15955 by mean of 7215. It was noted that CIAV antibody titers were significantly higher in flocks with viral isolates compared to flocks without (Table 3)

DISCUSSION

The results confirm the presence of CIAV in the examined broiler chickens. The present study is the first trial to isolate and identify CIAV infection in the examined broiler chicken flocks. CIAV infection is usually diagnosed by isolating the virus or by detecting virus specific antibody. Virus isolation is time consuming and the commonly methods are chicken embryos and chicks inoculations (Yuasa *et al.*, 1979).

The inoculated chick embryos showed no significant changes in the first and second passage but mildly observed in the 3rd in form of general less characteristic and diagnostic inflammations. The production of lesions and yield of the virus may require more serial passage for adaptation and propagation. The tissue harvest of egg embryos did not agglutinate chicken erythrocytes which consider a preliminary differential diagnosis of CIAV from NDV or avian influenza viruses. The failure of precipitation with positive antiserum might due to the specificity of the only available CIAV antiserum that works with immunofluorescence or immunoperoxidase. Inoculated chicks reacted differently from one flock/sample to the other, namely, clinical signs were observed with 5 samples (23%), mortalities in 2 (4.5%), but the lesions of undersized thymus and BF, edematous liver and spleen as similar for CIAV infection were found in 9 SPF chicks groups (41%) with or without symptoms. Although the mortalities were low and changes were less prominent but

were present and distributed on the target organs of the CIAV. These findings considered a specific for the presence of the virus in the chicken tissues. They might indicate and explains the variable pathogenecity of the virus isolates. However, other associated unknown virus content of the inocula as the samples were obtained directly from the field might play a related role in the development of such changes. The presence of CIAV specific antibodies of 276 and 907 in chicks on days 14 and 21 respectively was definitive confirmatory evidence on the presence of the infectious CIAV and in similarity and accordance with that reported by (Ledesma *et al.*, 2001). Propagation and reproduction of virus isolates by more serial inoculation and passaging into chicks that did not performed in this study is required and in agreement with (Zhou *et al.*, 1997) who's reported lesions in 60% inoculated chicks. However, lesions known as typical for CIAV clear hemorrhages on muscles were not reported here and in accordance with others concerning the different locations, conditions and virus strains (Lucio *et al.*, 1990).

Several studies of the isolation and identification of CIAV from naturally occurring infections have been reported (Yuasa *et al.*, 1979; Chettle *et al.*, 1989; McNulty *et al.*, 1989b; Zhou *et al.*, 1997). Most these reports described clinical and post mortem syndromes similar to that reported in this work. Nine isolates were preliminarily obtained from the 22 sampled flocks suspected of CIAV and in spite of the little facilities and low sample passaging. This coincides with other reports concerning the low incidence of successful virus isolations even if the samples came from flocks that were clinically and serologically suspicious for CIAV infection (Bulow and Schat, 1997; McNulty, 1991). Moreover, virus tropism and pathogenesis might be considered. Recently, the ideal media for isolation of the CIAV is cell lines such as (MDCC-MSB1) (Yuasa *et al.*, 1983) that did not use in this study. However, use of cell lines for propagation of such isolates probably will yield more virus antigens that can further be identified.

ELISA was efficient qualitative and quantitative assay for detection and monitoring of chickens to CIAV infections. The flock considered positive when at least one sample positive have. Frequent natural exposure of broilers to the virus excreted via faces or others takes 1-2 weeks until most birds have been seroconvert with long lasting antibody.

Maternal antibody can provide passive immunity for the progeny, no previous isolation and /or serosurvey reported antibodies against

CIAV, neither vaccination nor other preventive measures to CIAV are in practice. Therefore, the seropositivity of broilers is related to their field infection with CIAV or maternal transfer either in closed SPF or opened flocks. In addition, this explains lower productivity and inadequate vaccination responses in various broiler farms all around and in agreement with (McNullty *et al.*, 1991).

Most chicken's flocks 21 of 22 showed significant overall sero-conversion to CIAV (95%) by percentages ranged from 0% to 100%, while among birds were 375/467 (80%) that was higher in older broilers chickens than younger within the susceptible age. The log of antibody titers among the flocks ranged from 1.855 to 4.203 by titers of 72 to 15955 by mean of 7215 The serologic results showed that chickens had humoral antibody, confirmed the presence and wide spread of CIAV infection and in concordance with the findings reported by (Todd *et al.*, 1990b; Dren, *et al.*, (1996; Cardona *et al.*, 2000a).

A few serologic tests were used to detect and assess antibodies to CIAV by serum neutralization (SNT) (Jorgensen, 1990), indirect immunofluorescence (IIF) (Yuasa *et al.*, 1985). The used ELISA showed high sensitivity (97.5%), specificity (98%), suited well for serological testing of sufficient numbers of chicken sera and gave clear-cut positive reactions. The CIAV antibodies in the sera obtained either from field chickens (100%) or inoculated ones (32% to 45%) was positively correlated with the owners complains.

Conclusion: CIAV was isolated from the examined broiler chickens and identified by the serologic survey. Limitations of CIAV infection depends up on biosecurity measures control of the co-infection with lymphocidal agents, immunization using the inactivated vaccines of broiler breeders at weak 12 of age and prior to the onset of lay to assure better immunity for the progeny during their first weeks of life. Vaccination minimizes the vertical transmission and can prevent further virus dissemination.

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Table (1): The sampling size scheme from broiler chickens of various ages and flocks:

Flock No	Age / week	No. of samples / flock	Flock No	Age / week	No. of samples / flock
1	1	8	12	4	24
2	3	22	13	2.5	23
3	4	20	14	6	34
4	2.5	29	15	2.5	20
5	3.5	29	16	4	22
6	2	23	17	5	25
7	3	17	18	4	26
8	2	18	19	2	15
9	4	21	20	5	26
10	3	25	21	3	16
11	4	14	22	4	20
Total No of sampled flocks = 22					
Total No of sampled birds /all flocks = 467					

Table (2): Development and measurement of CIAV specific antibody titers in 2 days old chicks post inoculation with field tissue samples supernatants:

No. sample/flock	Signs	Lesions	Mean Ab day 14	Mean Ab day 21
1	-	*	0	+/613
2	-	-	0	0
3	+/+	*	0	0
4	-	-	0	0
5	-	-	0	0
6	+	*	+ / 378	+/1050
7	-	-	0	0
8	+	*	+/217	+/1193
9	-	-	0	+/ 217
10	-	-	0	0
11	-	*	0	0
12	-	*	+/217	+/1211
13	-	-	0	+/ 583
14	-	-	+/173	+/ 583
15	+/+	-	0	0
16	-	*	0	0
17	-	*	+/319	+/ 1352
18	-	-	+/43	+/967
19	-	-	0	0
20	-	-	0	0
21	+	*	+/583	+/1301
22	-	-	0	0
Mean of ELISA antibody titers			276	907

(-): No signs and/ or mortalities +: Mild signs appearance

+/+: Signs followed by mortalities; *: Gross lesions on the inoculated chicks on necropsy.

0: No antibody positivity; +/#: Antibody positivity / mean of antibody titers by KPL ELISA.

Table (3) Prevalence and frequency of ELISA antibody seropositivity and titers to CIAV in field sera from broiler chickens of various ages and flocks:

Flock No	Age / week	No. of samples	No. of positive	% of positive	Flock No	Age / week	No. of samples	No. of positive	% of positive
1	1	8	0	0 %	12	4	24	18	75%
2	3	22	17	77 %	13	2.5	23	18	78%
3	4	20	18	90 %	14	6	34	31	91 %
4	2.5	19	15	79 %	15	2.5	20	13	65 %
5	3.5	29	22	76 %	16	4	22	15	68 %
6	2	23	11	48 %	17	5	25	20	80 %
7	3	17	17	100 %	18	4	26	26	100 %
8	2	18	14	78 %	19	2	15	12	80 %
9	4	21	20	95 %	20	5	26	20	77 %
10	3	25	23	92 %	21	3	16	13	81 %
11	4	14	14	100 %	22	4	20	18	90 %
Total					22	1-6	467	375	80%
No positive flocks					21/22 (95%)				
Mean of ELISA antibody titer					7215				

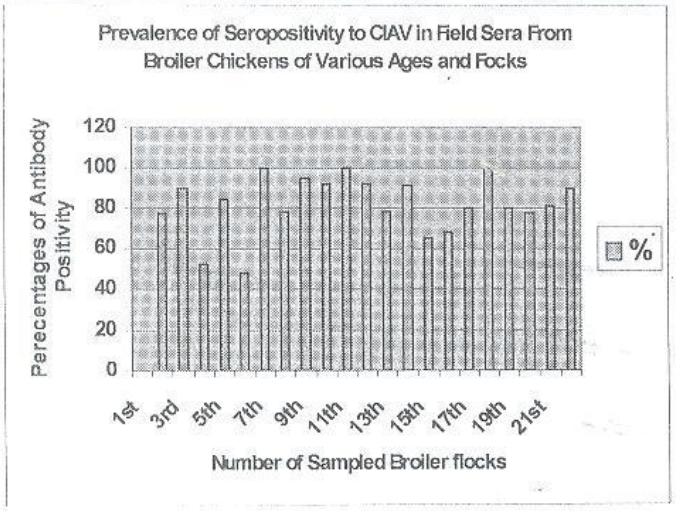

Figure (1): Prevalence of CIAV specific antibodies in broiler chicken's sera.