Egytptian J. Virol. 5 (1): 304-321 (2008)

Immune response to a live attenuated chicken anemia virus (CAV) vaccine. the absence of virus shedding.

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An assessment of the performance of a live attenuated (CAV) vaccine following administration to broiler breeder hens in eight houses at 11 weeks of age. Antibodies to (CAV) in adult hens and maternally derived antibodies to (CAV) in progeny chicks were assayed by ELISA. Vaccinated dams showed a high level of antibody to (CAV) followed by moderate level for up to 50 weeks of age. In addition progeny chicks showed a detectable level of maternally-derived antibody. New castle Disease Virus (NDV) antibodies were monitored in adult hens during the first 22 weeks of their age using Haemagglutination inhibition (HI) technique. Dams showed sufficient of antibodies to (NDV) through this period indicating that the vaccinated hens were immunopotent. No sign of immunosuppression in progeny chicks was detected by measuring (NDV) HI antibody titers pre and post lasota vaccination and by vaccination challenge experiment, more over no (CAV) shedding was observed in progeny chicks at early stage of breeding period. In this stage CAV shedding was recorded to be most using CAV VPI PCR assay. These results support the evidence that vaccination of breeders flock with the live attenuated (CAV) vaccine could be an effective means of control of chicken anemia virus induced clinical disease.

Key words:

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(Received March 2008)
(Accepted May 2008)

INTRODUCTION

Chicken infectious anemia (CIA) a disease of young chickens, is caused by a unique small virus circular DNA (**Gorgo et al., 1987**) (9). CAV was found to enhance the pathogenicity of a range of co-infecting agents (**Bulow et al., 1983**) (2). Immunosuppression caused by CAV thus causes serious economic losses in commercial poultry production (**Novak et al., 2001**) (13). CAV is known to spread horizontally through contact between chicks (**Yassa et al., 1980**) (21) or vertically from breeder to their progeny (**Yassa and Yoshida, 1983**) (22). High levels of CAV ELISA antibodies in breeder flocks protect against vertical transmission and outbreaks of CIA in progeny (**Brentano et al., 2005**) (1). Maternally derived antibodies to CAV are known to protect chicks from infection (**Otaki et al., 1992**) (14). Outbreaks of Blue wing disease (BWD) were reported in progeny flocks from broiler breeders with the exception of those that had been vaccinated (**Engstrome, 1999**) (6). Clinical disease of CAV is rare today because of widespread of practice of vaccinating breeders with the inactivated and live attenuated CAV vaccines (**Franz and Carol, 2003**) (7). However, live attenuated CAV vaccines have the possibility of reversion to virulence (**Page Mainte et al., 1997**) (15), and reports have surfaced that the pathogenicity of attenuated CAV viruses could be restored after 10 passages in young chickens, so the irreversible attenuation of CAV is proving difficult (**Todd et al., 1995, 1998**) (18,19). In addition, recent reports have demonstrated that an attenuated CAV vaccine strain induced anemia and lesion in the lymphoid organs of young chicks (**Hussein et al., 2003**) (11). These reports have instigated our investigation in the effect of field application of a commercially available live attenuated CAV vaccine on the immune response of broiler breeders to that CAV vaccine and to vaccination against other important viral pathogens. We have also investigated the shedding of CAV from vaccinated broiler breeder flocks to their progeny at the early stage of the breeding period using the highly sensitive PCR technique against CAV VP1. In addition, we attempted to detect any signs of clinical immune suppression in progeny chicks from vaccinated

commercial breeder broiler flocks using an NDV vaccination challenge experiment.

MATERIALS AND METHODS

Materials:

Birds:

- Broiler breeder Hubbard chickens housed in close system (house numbers 1, 2, 3, 4, 5, 6, 7 and 8) 5000 birds per house were vaccinated with live attenuated CAV vaccine at 11th week of age before egg laying, after rearing period, chickens were pooled, transformed to new farm and then redivided into houses for preparation to the breeding period.
- 190 one day old Progeny chicks from the above mentioned breeders were divided into three groups, group(V1) 90 chicks from 31 weeks old breeders (early stage of breeding period), group (V2) 50 chicks from 38 weeks old breeders (intermediate stage) and group (V3) 50 chicks from 46 weeks old breeders (late stage).
- 50 one day old progeny chicks from CAV unvaccinated dams
- 18 one day old SPF chicks.

Virus:

Virulent standard strain to NDV contains 106 EID50, dose of NDV.

CAV ELISA antibody test kit (Synbiotic,USA)

Material used for PCR assay

a. Primers (Bio Basic Inc.) CAV1 and CAV2

CAV1 sequence 5' GACTGTA-AGATGGCAAGACGAGCTC3'
CAV2 sequence 5'GGCTGAAG-GATCCCTCATTC3'

b. QIAamp SPIN Columns used for extraction of CAV DNA from liver, bone marrow samples
c. Reverse- it^{TM} one-step RT-PCR kit (AB-gene, UK)
d. Molecular weight marker, 1 kb DNA ladder (AB-gene) which consists of 13 DNA fragments which size of 250, 500, 750, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000

Method:

- Breeders were Routinely Vaccinated with NDV Vaccines at age of 1 day, 12 days, 23 days,

8 weeks, 11 weeks, 16 weeks, 19 weeks and 21 weeks

- ND HI Antibody GMT were Monitored in Randomly selected Breeders in the (8) houses , 14-16 serum samples per house at ages of 4 days , 6 weeks , 8 weeks and 11 weeks before CAV vaccination and at age of 14 weeks , 17 weeks , 20 weeks and 22 weeks after CAV vaccination using HI technique .
- ND HI antibody GMT were measured in progeny chicks from CAV vaccinated breeders at 31 weeks of age (early stage of breeding period), serum samples of chicks were collected at age of 2 days , 9 days , 15 days before lasota vaccination which was applied at 16 days of age . Also serum samples were collected at age of 22 days after lasota vaccination, 8 – 12 samples per age period.
- Protection against NDV challenge (at 3 weeks post lasota vaccination) In the progeny chicks of breeders at early stage of breeding period which were divided into two groups , group V1 (30) chicks vaccinated with lasota eye drop vaccine and group V2 (10) chicks kept unvaccinated . also we used 20 chicks from different origin not vaccinated with lasota (group C) all groups (V1, V2 and C) were challenged with virulent standard strain of NDV (10^6 EID50 /bird) at 37 days of their age (3^{rd} week post lasota vaccination in case of group V1) . Challenged chicks were observed for 10 days post challenge mortality, morbidity and symptoms due to NDV infection were recorded. Protection percentage was calculated in the three groups V1, V2 and C.
- CAV ELISA antibody titers were monitored in the serum of randomly selected breeders in the (8) houses at age of 4 days and 11 weeks before CAV vaccination and at 14 weeks, 16 weeks and 19 weeks of age after CAV vaccination. 11-13 serum samples per house. chickens were pooled, transferred and then re-divided into houses for preparation to the breeding period then randomly selected chickens at ages of 33 weeks , 37 weeks , 42 weeks , 46 weeks and 50 weeks were bled. 8-10 chickens per age period then collected sera were used in estimation of CAV antibody titers using ELISA technique.
- CAV ELISA maternal antibody titers were monitored in

the three groups of progeny chickens V1, V2, V3 from CAV vaccinated dams at 31 weeks, 38 weeks, and 46 weeks of age respectively through the first few weeks of their age. also (50) one day old chickens from CAV unvaccinated 44 weeks – old – breeders (group UV) from different origin and (18) one day old SPF chicks (group F) were used as a control groups . Randomly collected 7 – 10 chicks were bled from each group.

Detection of the possible CAV shedding from progeny chicks of CAV vaccinated breeders at early stage of breeding period using CAV PCR assay individual liver and femoral bone samples were collected from five 2 days old progeny chicks and eight 9 days old chicks from 31 weeks old CAV vaccinated dams. Collected samples from chicks at the same age were pooled grinded and processed with addition of antibiotics then three times of repeated freezing and throwing were done, homogenate was centrifuged CAV, DNA was extracted from the processed sample using QIAamp DNA mini kit one hundred and eighty (180) ul of PCR master mix was prepared for 10 samples by mixing 100 ul 2x RT-PCR master mix, 16 ul primer mix (8 ul CAV1 and 8ul CAV2) and 64 ul nuclear free water in PCR tube. Total PCR master mix per sample was 18 ul. Two ul template DNA (sample) was dispensed in the tube to obtain final volume of 20 ml then PCR master mix was put into the thermo cycler in which the mixture was incubated at 94 °c for 5 minutes then subjectedto 50 cycles of 94°c30 seconds, 55 °c for 30 seconds and 72 °c for 30 seconds then subjected to 72°c for seven minutes.

Electrophoresis:

* (0.6) g agarose was weighed and added to 50 ml 1x Tris acetate EDTA.
* The mixture was put in the microwave till it was completely dissolved.
* The agarose solution was left to cool till 55 °C, then Ethedium bromide was added in a concentration of 0.5 µg/ml and mixed by swerling.
* The mixture was poured in the gel casting tray fitted with a comb to a thickness of 3-5 mm and left at room temperature for solidification for 20-30 minutes,

after solidification, the tray containing the gel was moved into the electrophoresis tank, covered with electrophoresis running buffer (1x TAE buffer) and the comb was removed.

* Eighteen (18) µl of each sample was mixed with 2 µl 10X loading dye to obtain 20 µl dye sample mixture. Samples (20 µl) as well as the molecular weight marker (5-7 µ) were dispensed in wells of the agar gel.

* The electrophoresis chamber was covered and the apparatus lid was connected to the power supply electrophoresis was performed at 90 volts for about 30 minute then the gel was examined using short wave UV light using the transiluminator and photographed using poloraid camera and film.

minutes in a dark place.

RESULTS

- The immune response of CAV vaccinated breeders group (V) to NDV pre-CAV vaccination period and post CAV vaccination period

Table (1) showed that log_2 of the NDV HI antibody GMTs of breeders in the eight houses at 14 weeks, 17 weeks, 20 weeks and 22 weeks of age after CAV vaccination were high and homogenous. The overall titres of the different houses are homogenous, specially after application of vaccination program adapted by the owners.

Breeder age	Vaccination with CAV vaccine	Newcastle disease HI Geometric mean titre (GMT) (ND HI range) expressed as log2							
		House No.							
		1	2	3	4	5	6	7	8
4 days	Pre CAV vaccination	2.3 (1-4)	2.6 (1-5)	4.4 (3-6)	3.2 (1-5)	6.0 (4-8)	5.5 (3-7)	6.7 (6-8)	5.6 (3-7)
6 weeks		6.1 (3-8)	3.7 (1-6)	3.8 (1-8)	3.9 (2-8)	2.8 (1-6)	2.6 (1-8)	2.3 (2-7)	2.3 (1-7)
8 weeks		6.5 (5-9)	6.0 (4-8)	5.0 (2-8)	5.0 (0-8)	5.8 (1-9)	5.6 (3-7)	6.3 (5-8)	5.6 (3-8)
11 weeks		5.8 (1-8)	5.7 (3-8)	6.3 (4-9)	6.2 (1-9)	5.8 (4-7)	6.0 (4-8)	5.8 (4-9)	4.9 (2-8)
14 weeks	Post CAV vaccination	9.2 (6-11)	8.3 (7-10)	8.3 (5-11)	8.5 (7-10)	9.4 (8-11)	8.4 (5-10)	8.0 (5-11)	7.7 (3-11)
17 weeks		7.1 (5-9)	7.3 (5-8)	7.7 (5-10)	7.0 (4-11)	8.4 (6-11)	7.2 (5-9)	7.8 (6-10)	7.5 (6-10)
20 weeks		9.7 (9-11)	9.5 (8-11)	8.5 (8-9)	8.8 (8-10)	8.4 (6-10)	8.7 (6-12)	9.1 (7-10)	10.3 (7-12)
22 weeks		8.3 (6-10)	8.2 (7-10)	7.7 (7-9)	7.4 (5-10)	9.4 (8-10)	7.5 (6-10)	7.9 (5-10)	8.1 (6-11)

Table (1)

- NDV HI antibody GMT of progeny chicks of breeders at early stage of breeding period before and after lasota vassination.

Table (2) showed that the ND HI GMT in progeny chicks from 31 weeks old CAV vaccinated dams decreased gradually. On the other hand, ND HI GMT increased at 22 days of age seven days post LaSota vaccination.

Chicks Age	LaSota vaccination	ND HI antibody GMT *	ND HI range	No. of chicks	SD
2 days	Pre-vaccination	8.32	7-9	8	0.91
9 days		5.96	3-9	11	1.6
15 days		4.97	3-7	10	1.54
22 days (7 days post LaSota vaccination)	Post Vaccination	7.28	5-9	12	1.37

Table (2)

- Protection against NDV challenge in chicks from CAV vaccinated breeders following NDV vaccination:

Table (3) shows that the challenge of progeny chicks of 31 weeks old CAV vaccinated breeders resulted in only 3.4 % mortalities among the NDV HB1 and LaSota vaccinated group (V1). However, there were 100 % and 80 % mortalities in HB1 vaccinated group (V2) and in unvaccinated group (C), respectively

Group of chicks	Progeny chicks		Negative control chicks
	V1	V2	Commercial (C)
Vaccination with ND vaccine	Vaccinated with HB1 in hatchery and with LaSota at 16th day of age	Vaccinated with HB1 in hatchery	Unvaccinated with ND vaccine
No. of chicks at time of NDV challenge	30	10	20
No. of deaths at first ten days post NDV challenge	1	10	16
Protection percentage	96.6 %	0 %	20 %

Table (3)

- CAV ELISA antibody GMT for breeder broiler chickens (group V) during the rearing period pre and post CAV vaccination:

Table (4) shows that the overall CAV ELISA antibody GMTs of breeders in the different houses are high and homogenous at 3 weeks, 5 weeks and 8 weeks post CAV vaccination, respectively if they compared with the titres at 4 days and 11 weeks of age pre-CAV vaccination SD values were low in relation to the corresponding ELISA GMT at the post CAV vaccination period if compared with SD values at the pre-CAV vaccination period. CV% values are low and homogenous at the post CAV vaccination period if compared with the pre-vaccination period.

CAV vaccination	Breeder chickens age		House Number							
			1	2	3	4	5	6	7	8
Pre-vaccination	4 days	GMT	2088	4076	5107	2113	4503	3972	2065	4394
		SD	2059	1230	574	2356	1358	1440	1867	1254
	11 weeks	GMT	5760	4731	1980	2944	4708	16	4677	3568
		SD	1742	1335	2966	1363	2078	1047	2204	1690
Post vaccination	14 weeks	GMT	6282	6658	7244	5791	6889	5396	7930	7841
		SD	1707	1273	1441	2512	2124	3026	4063	3195
	16 weeks	GMT	8270	8001	9865	10381	10161	10217	10084	12353
		SD	2870	3122	2050	4015	2563	2119	5260	4876
	19 weeks	GMT	10234	8549	10215	10065	9525	9877	7687	9883
		SD	2645	1676	1916	3555	2882	2179	2952	2503

Table (4)

- CAV ELISA antibody GMT for breeder broiler chickens group (V) during the breeding period post CAV vaccination:

Table (5)revealed that CAV vaccinated breeder chickens at 11 weeks of age showed a high detectable level of CAV ELISA antibody GMTs. At the period between 33 weeks and 50 weeks of age which were nearly constant.

	CAV ELISA antibody GMT				
	Age of broiler breeder chickens (weeks)				
	33 weeks	37 weeks	42 weeks	46 weeks	50 weeks
GMT	6160	6235 *	5937	5219 *	5631
No. of birds	10	10	10	8	10
SD	1831	1343	1585	682	1374

Table (5)

- CAV ELISA antibody GMT for breeder broiler chickens (Group V) pre and post CAV vaccination:

Table (6) and Fig. (1) show that CAV ELISA antibody titre detected at 4 days of breeder's age then waned with time but still detected at eleven weeks of age. The CAV antibody titre highly shotted to high level at the post CAV vaccination period till reached its maximum level at 5 weeks post vaccination and continued nearly constant till 8 weeks post vaccination at the rearing period, then the antibody titre decreased slowly till reached moderate levels at the breeding period and remain nearly constant at the period between 31 weeks and 50 weeks of age.

- CAV ELISA maternal antibody GMT in progeny chicks from CAV vaccinated breeders, CAV unvaccinated breeders and SPF breeders:

	CAV Vaccination									
	Pre CAV vaccination		Post CAV vaccination							
Breeder Age	4 days	11 weeks	14 weeks	16 weeks	19 weeks	33 weeks	37 weeks	42 weeks	46 weeks	50 weeks
GMT	3324	1938	6698	9836	9462	6160	6235	5937	5219	5631
No. of birds	90	90	90	90	90	10	10	10	8	10

Table (6)

The result in Table (7) revealed that the 2 days old progeny chicks from CAV vaccinated parent breeder chickens showed a detectable level of CAV ELISA maternal antibodies GMT. Maternal antibodies in progeny chicks from 31 weeks old dams was higher than that in progeny chicks from 38 weeks old dams while the CAV ELISA maternal antibody GMT in progeny chicks from 46 weeks old dams was the lowest. On the other hand, 2 days old progeny chicks from CAV unvaccinated breeder chickens, also, showed a detectable CAV ELISA maternal antibody GMT, while SPF chicks did not show a detectable CAV ELISA maternal

CAV vaccination →	CAV vaccinated dams before egg laying						CAV unvaccinated dams			
Age of dams (group) →	31 weeks (V1)		38 weeks (v2)		46 weeks (V3)		Commercial 44 weeks (UV)		SPF (F)	
Age of chicks	No. of chicks	GMT (SD)	No. of chicks	GMT (SD)	No. of chicks	GMT (SD)	No of chicks	GMT (SD)	No of chicks	GMT (SD)
2 days old	8	3633 (998)	9	3509 (1268)	7	2877 (970)	9	3521 (954)	-	ND
9 days old	10	1443 (474)	9	1786 (684)	9	3233 (740)	7	1637 (409)	2	196 (521)
21 days old	10	890 (458)	8	1395 (580)	9	1333 (989)	9	1358 (1155)	3	387 (750)
30 days old	10	775 (477)	-	ND	10	1704 (795)	10	2175 (1691)	7	448 (386)

Table (7)

antibody GMT.In addition, CAV ELISA maternal antibody GMT woned gradually along the first 30 days of progeny chick's life from 31 and 38 weeks old CAV vaccinated breeder chickens. On the other hand, GMT in progeny chicks from CAV unvaccinated breeder chickens increased at 30 days of their age.

- Detection of CAV shedding in chicks of CAV vaccinated breeders at early stage of the breeding period:

CAV VP1 detection in pooled samples from 2-days-old and 9-days-old chicks of 31 weeks-old breeders. This experiment was conducted for testing of CAV DNA in the pooled clinical samples of chicks from vaccinated breeders. Pooled liver and bone morrow samples from two-days old chicks (n=5) and 9-days old chicks (n=8) from 31 weeks old broiler breeders were extracted. Two microliters from each extract were tested together with a negative control and a positive control. CAV DNA was detected in the extract of the control positive tissue culture vaccine (Photo (1)

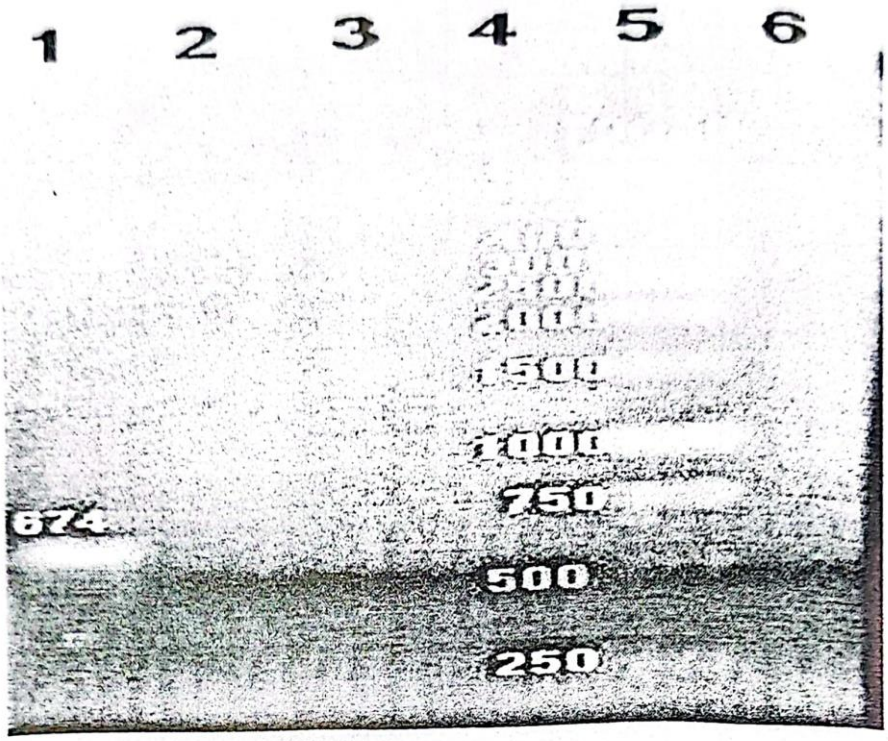

Photo (1) : CAV DNA was detected in the extract of control positive tissue culture vaccine. lane 1. No CAV DNA was detected in extracts from the SPF allantoic fluid, the pooled samples from 2-days-old chicks or the pooled samples of 9-days-old chicks, lanes 2, 3 and 4, respectively. The test blank was negative, lane 6. The 1 kpb ladder from AB gene is in lane 5.

DISCUSSION

Investigation in this work did not provide any evidence to support the hypothesis that CAV vaccination with a live attenuated CAV vaccine produce immune suppression when used as recommended.breeders responded well to NDV vaccination and continued to develop the normal response pattern following CAV vaccination at 11 weeks. A satisfactory immune response to NDV was present in the 8 breeder houses investigated (Table 1). Vaccination of the progeny chicks with NDV vaccines, the HI titer patterns developed normally (Table 2). In agreement, our challenge experiment showed that 96.6% of the HB1 and LaSota vaccinated progeny chicks were protected against NDV challenge (Table 3). Results in tables (1 and 2) if compared with result of **Danial (1996) (4)** who studied the immunosuppressive effect of CAA and he found that ND HI antibody titres in CAV inoculated chicks ranged between 4-512 while in non-inoculated groups was 16-1024 at different time post CAV vaccination. Finally, we concluded that neither parent breeders (Table 1) nor their progeny (Table 2) immunosuppressed. This result does not necessarily contradict reports that the administration of the live attenuated CAV vaccine at 1 day of age caused clinical priplem (**Hussein et al., 2003**) **(11).** In this work, the application of the vaccine was done according to the manufacturer's instructions at 11 weeks of age.The finding that 100% of the chicks vaccinated only using HB1 succumbed to challenge NDV is not unexpected and are in agreement with other challenge experiments conducted using a single vaccination dose administered at 1 day old **(Villegas, et al., 1977) (20).** The development of a protective immune response required the LaSota booster vaccination, since the protection against NDV depends on the presence of NDV antibodies against the challenge virus **(Giambrone, 1981) (8).** Our investigation indicated that the live attenuated CAV vaccine administered in 11 week-old breeding birds was immunogenic to the vaccinated birds. There was a substantial increase in CAV ELISA reactivity following a single application of the vaccine. The vaccine application resulted in a uniform development of relatively

high levels of CAV ELISA antibodies in the vaccinated breeder flocks compared to the control unvaccinated flocks (**Pages Mainte et al., 1997**) **(15)** and compared to the pre-vaccination results,as demonstrated by relatively low SD values post-vaccination (Table8). These results are in accordance.with other research conducted on the use of CAV vaccines (**Page-Mainte, et al., 1997; Brentano et al, 2005**) **(15,1).**Relatively high CAV ELISA antibody titers were detected in vaccinated flocks up to 39 weeks post vaccination, which is the duration of our experiment, (Table 5,6). **Pages-Mainte, et al., 1997**, reported detectable CAV ELISA titers up to 40 weeks of age. The persistence of the CAV antibody titers is most probably attributed to the continuous natural exposure to the CAV in farms, which in turn boosts the antibody level in the immunized birds (**Pages-Mainte, et al., 1997**) **(15).**The detection of CAV ELISA antibodies in 4 day-old chicks is a result of maternal transfer of CAV antibodies in yolk from grandparents (Table 4). The grandparents of the breeders in our experiment were vaccinated using the same CAV vaccine used in our experiment. Thus, CAV vaccination stimulates protective immunity in parent breeders that would be transferred to progeny and protect them from CAV infection early in their life (**Rasales, 1999, Franz and Coral, 2003**) **(16,7).**The antibody levels detected in progeny chicks at 2 days of age using the CAV ELISA indicated a slow decrease in the amount of material antibodies transferred to the offspring with the increase in the dam's age (Table 7). This is in agreement with the results of antibody titers from breeder dams (Table 6). On the other hand progeny chicks from CAV unvaccinated breeders showed a detectable CAV maternal antibodies at hatch, which may be attributed to an infection in parent flocks during rearing. **Sabry et al., 1998 (17),** reported that maternally derived antibodies were present in the majority of day-old broiler chickens. We noticed that 21 days-old chicks from non-vaccinated dams exhibited an abnormal increase in the SD value (1155) despite the fact that the corresponding geometric mean was within the range of the vaccinated groups (1358) (Table 7). The subsequent increase in antibody titers at 30-days of age indicated that an exposure had happened

probably around the second week of age (Table 7).In other groups the CAV antibody titers waned along the first 30 days of the chick's life with a half life time approximately 7 days. This result agreed with **Otaki et al. (1992)(14).**It is important to consider that groups V3 and UV were housed close to each other and this explain why V3 antibody titers did not decline as expected between 9 and 30 days of age like V1. V3 could have experienced an exposure to the CAV from the environment early in the beginning of the experiment, yet the presence of protective antibodies from its dams resulted in a delay of seroconversion similar to that observed in UV **(Engstrom, 1999)(6).**Although our finding regarding the induction of immune suppression in vaccinated chicks led us to the conclusion that the vaccine use does not induce immune suppression, this work does not dismiss the possibility that future mutation of the vaccinal strain may lead to clinical and immunological problems in the future. There is a need to continue this line of investigation and follow up of vaccine usage consequences in the future.

We were not able to detect any virus shedding in the samples that were collected from chicks of vaccinated breeders This could be attributed to the presence of sufficient protective immunity in the vaccinated breeders. The detectable level of maternal antibodies have been reported to block vertical shedding of CAV **(Hoop,1992;Malo and Weingarten, 1995; Dren et al., 2000)(10,12,15).** Also, replication of CAV could not be demonstrated in chickens that were positive for maternal antibodies to CAV **(Carrie et al.,2003)(3).**

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