

The Prevalence of Infectious Bronchitis (IB) Outbreaks in Some Chicken Farms. I. Spotlight on the Status of IB Outbreaks in Some Chicken Flocks.

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Abstract: Twenty five isolates of IBV were isolated from 36 broiler, layer and breeder chicken farms collected from 13 governorates during 2 years started from January 2003. Sixteen farms were vaccinated against IB, and nine farms were not vaccinated. The cardinal signs of the disease in layers were drop in egg production, with watery albumen, inferior (pale-misshaped shell) eggs, un-noticed respiratory distress and pectoral myopathy, and those in broilers were respiratory distress, renal urate deposition and death beyond four weeks of age (late mortality). The viruses were isolated and identified by chicken embryo, and CEK cell culture inoculation. [Journal of American Science 2010;6(9):57-70]. (ISSN: 1545-1003).

Keywords: Infectious Bronchitis (IB); Chicken; Farms; Flocks

1. Introduction

Infectious bronchitis virus (IBV) is the causative agent of the famous disease internationally known as infectious bronchitis (IB), that causes highly economical losses in poultry. Infectious bronchitis is one of well known respiratory and urogenital disease of chickens (Cavanagh and Naqi, 1997), all over the world since 1931, but specifically in 1954 in Egypt. It is well known that the primary tissue of IBV infection is the respiratory tract, though some strains also replicate in the kidney and oviduct, causing nephritis and reduced egg production; respectively. IBV has a constant threat to the poultry industry because of the isolation –now and then - of new variant serotypes of the virus even from vaccinated flocks of different immune status (Gelb, 1989; Wang and Tsai, 1996). Till now, more than 60 serotypes or IBV variants have been identified worldwide (Ignjatovic and Sapats, 2000; Yu et al., 2001), against which little or even no-cross protection existed. Because of this fact, determining and up dating the exact serotypic identity of field strains prevalent in poultry farms in Egypt is very essential for selecting the effective vaccine capable to overcome the problem of IB disease in Egypt.

For an effective vaccination program, the isolation and identification of IBV isolates are important because vaccines are selected on the basis of the serotypes present in specific geographic areas (Yu et al., 2001).

In Egypt, IB was first described by Ahmed (1954), subsequently several reports emphasized the prevalence of the disease (Ahmed, 1954; Eissa et al., 1963; Ahmed, 1964 ; Amin and Mustaggar, 1977 and El-Kady, 1989). Massachusetts (Mass) type live

attenuated vaccine (H120) as well as inactivated oil emulsion vaccine are applied to prevent and control the incidence of the disease.

The aim of this study was to investigate the prevalent IBV in Egypt and their evolutionary relationship. The present work particularly interested to know whether the recently isolated Egyptian IBV strains which escaped from vaccine- elicited immunity were newly introduced in the chicken population or arise by mutations of circulating Egyptian IBV strains .This is important for implementation of control measures especially for the future vaccination strategies

2. Material and Methods

1. Viruses of IB:

1.a. Field isolates: Affected and freshly dead birds were collected from 36 chicken farms showing symptoms suspected to be IBV infection. The collected birds were killed, necropsied and examined for gross post mortem (PM) lesions. Specimens for IBV isolation included trachea, lung, kidney and cecal tonsils were collected under aseptic condition according to Jose et al., (2000).

1.b. IB AGP antigen: Were supplied from, Charles River Laboratories, SPAFAS. Co., Lot. No. 536216; in a lyophilized form and reconstituted by addition of 1.0 ml sterile PBS buffer (according to the direction of manufactures). Reconstituted antigen stored at -20°C till used as positive control in AGPT of CAM homogenate of the inoculated SPF eggs.

2. Serum:

Positive infectious bronchitis virus precipitating antiserum: Antiserum was supplied

from Holland, Diventure. IBV AGP/GDT antiserum, Lot No. 20102-140400. in a lyophilized form and reconstituted by addition of 1.0 ml sterile PBS buffer (according the direction of manufactures). Reconstituted antiserum stored at -20°C till used in detection of IBV antigen in the CAM homogenate of the inoculated SPF eggs by AGPT.

3. Experimental hosts:

3.a. Fertile chicken egg: The fertile chicken eggs used through the present study were Specific Pathogen Free (SPF) eggs originated from Nile SPF (Koom Oshiem, Fayoum, Agriculture Research Center-Ministry of Agriculture). The fertile chicken eggs were used for isolation of IBV by egg inoculation, preparation of chicken embryo kidney cells (CEK).

3.b. Cell culture: Monolayer cultures of primarily chicken embryo kidney cells (CEK) were prepared from the kidneys of 19-20 day old specific pathogen free (SPF) chicken embryo according to (Villegas and Purchase, 1990).

4. IBV isolation:

4.a. Preparation of samples for IBV isolation (Jose et al., 2000): The collected organs (Trachea, lung, kidney, cecal tonsils) were washed in sterile 0.85% saline, and then frozen at below -10°C. After thawing, the tissue homogenates (10% W/V) were prepared in sterile saline 0.85% containing 1000 IU/mL penicillin, 1.0 mg/ml streptomycin. By disrupting organs using sterile mortar and pestle, the homogenates were then centrifuged at 3000 rpm for 10 min, and the supernatant was further passed through 45 µm membrane filter. Sterility of the inocula was checked pre-inoculation by culturing on nutrient agar and sabouraud's dextrose agar. These materials were examined for presence of IBV by passage in embryonated eggs.

4.b. Specific Pathogen Free (SPF) embryonated chicken egg inoculation (Gelb and Jackwood, 1998): Five to eight 9-11-day-old SPF embryonated chicken eggs were used for inoculation of each sample via the allantoic sac route. 0.2 mL of the inoculum was inoculated per egg. On day 3 pi, survival embryos were killed and chorioallantoic fluid and chorioallantoic membrane (CAM) were harvested aseptically from inoculated eggs. Chorioallantoic fluid was tested for sterility to be free from bacteria and fungi by culturing on nutrient agar and sabouraud dextrose agar, and tested for haemagglutination (HA) reaction with 10% chicken red blood cells (CRBCs) (to exclude haemagglutinating agents). The harvest fluids were inoculated for two passages (2nd and 3rd) (some of IBV field isolates were not embryo-adapted and did not cause death or produce lesions

on the first passage (Gelb and Jackwood, 1998), so, further, 2 additional passages (4th and 5th) were performed, each in 5-8 embryos and observed for typical IB lesions, such as dwarfing and stunting (judged by weight if the difference was of 25% or more between infected and normal embryos of the same age, may be considered evidence of IBV infection, Anon, 1963). Chorioallantoic membranes harvest homogenates were tested in AGP test (Woernle, 1966) for evidence of IBV infection.

5. Agar gel precipitation test (AGP): The test was used to demonstrate the presence of IBV antigen in the harvests of chorioallantoic membranes (CAMs). The test was performed according to Chubb and Cumming (1972). Reading were taken 24-48 hours after filling with an oblique light in a dark room.

6. Isolation of IBV in chicken embryo kidney (CEK) cells:

6.a. Chicken embryo kidney (CEK) cell culture preparation: These cultures were prepared from kidneys of 19 to 20-day-old SPF chicken embryos according to Villegas and Purchase, (1990)

6.b. Inoculation of CEK cell culture by IBV isolates (Villegas and Purchase, 1990):

0.1 ml of IBV (allantoic harvest) at the level of 5th embryonic passage for each isolates (positive in AGP test), were inoculated into separate tissue culture plate, the plates rocked gently for evently distribution of the inoculum over the cell monolayer. Inoculated cultures were incubated at 37°C for 45 minutes to allow virus adsorption. The plates were rocked once or twice during incubation. 2ml of MEM contain 5% calf serum was added to each plate.

Plates were then incubated at 37°C with 0.5% CO₂ with daily observation for cytopathic effect (CPE) and the condition of the cells. If no CPE for up to 48-72 hours, re-passage was performed. For re-passage, the samples were harvested after 3 cycles of freezing and thawing, then collected and used for a second serial passage.

3. Results

1. Characteristic of IB outbreaks in poultry farms:

The present data represent prospective survey of the presence of IB disease in 36 chicken farms. The data collected from 13 governorates during 2 years started from January 2003 and involved different types of chickens, including broilers, layers and broiler breeders (table 1).

In broiler farms (table 2) out of 24 examined farms, 9 farms with history of previous vaccination

against IB which represent 37.5%, and 15 farms without history of vaccination, which represent 62.5%. The main clinical signs were difficult in breathing, tracheal rales, coughing, sneezing with or without nasal discharge, wet eye was observed and an occasional chick may have swollen sinus (Fig. 1A). Elevated mortality was observed beyond 4 weeks and persisted for the end of fattening period with range of 5-25%. A generalized weakness was observed, accompanied by depression. Feed consumption and body weight were markedly reduced. Soiled vent feather was recorded as accompanied by slight diarrhea or soft feces and wet litter (Fig. 1B). On necropsy, the trachea was congested with excessive amounts of mucous (Fig. 2). Casious exudate in trachea and its bifurcation as a plug was seen. Air sacs showed variable observations including cloudy, turbid with or without yellow casious exudates. Most of examined chicks were associated with pericarditis, perihepatitis and enteritis. Sometimes small area of pneumonia was observed. Some of the examined chicks revealed nephritis as swollen and pale kidneys, sometimes with tubules and ureters deposits with urates (Fig.3). In few cases, peticha of hemorrhage were seen on the mucosa of proventriculus with or without thickening of the musculature.

Clinical signs in replacement layers and breeders were less in severity, in the form of mild respiratory disease with coughing, sneezing and rales. Hens in production respiratory signs were unnoticed, but mainly decline in egg production was the common sign, which ranged between 8% and 30%. The start of egg production in some flocks retarded 3-5 weeks with unpeaking to the standard, also accompanied with eggs of smaller size (about 5%) and inferior shell and internal egg quality, were seen as soft – pale-shelled and misshapen eggs, and eggs with thin albumin (Figs 4 and 5). In majority of cases, production levels remains subnormal. In one recorded broiler breeder farm, fertility reduced to 77% (13% below standard). On necropsy of dead laying hens, oviduct length was reduced, and ovarian regression was noticed in some birds. Yolk material was often found in the abdominal cavity. One broiler breeder farm, exhibited pale and swollen deep pectoral muscles associated with gelatinous edema over the surface of the muscle. Bilateral myopathy affected both superficial and deep surface of the muscles (Figs. 6 and 7). 2. Trials of isolation and identification of IBV:

2.a. The influence of different IB virus strains on chicken embryos.

Samples of trachea, lung, kidney, and cecal tonsil were taken from chickens were prepared for egg inoculation. For each sample to be examined, five to eight 9-to-11-day-old (SPF) eggs were used. After 6 days of incubation, the eggs were examined for lesions indicative of IBV infection (dwarfing and curling of the embryo). The allantoic fluid was collected and tested for haemagglutination (HA) reaction with chicken red blood cells (CRBCs) (to exclude haemagglutinating agents). Uninoculated SPF eggs were always included as control of embryo size. Each sample was given four or five passage before being considered negative

Preliminary identification of suspected virus isolates as IB was done by an agar gel precipitation (AGP) test. The chorioallantoic membrane (CAM) were harvested from inoculated eggs for each sample at the level of third, fourth and fifth passage from both dead or chilled embryos, washed with sterile saline, grinded, freezed and thawed for several times, centrifuged at 3000 rpm for 10 minutes and the supernatant fluid were examined by AGP test against positive precipitating IBV antiserum for evidence of IB infection.

1. Results: revealed that 25 samples were positive for IB in agar gel precipitation (table 3 and 4).

2. In broiler farms incidence of the infection was recorded beyond 4 weeks of age (18.75%), at 5 weeks (37.5%), at 6 weeks (37.5%) and at 7 weeks (6.25%) (table 5).

3. Embryonic mortality within 2-6 days pi during five embryonic passage (table 6 and 7) revealed that some IBV field isolates were not embryo adapted and did not cause death or lesions in the first passage, therefore 5 passages were made before virus isolation attempt is considered to be negative.

4. Adaptation of IBV-field isolates to egg embryos by further passages up to the 5th passage, was associated with dwarfing as judged by reduction in percentage of infected embryonic weight by approximately 25% less than of non-infected embryo weight (table 8), hemorrhage cutaneous lesions, curled into spherical form with feet deformed and compressed over the head. Some embryos showed mesonephrous containing urates and thickened amnion covering the stunted embryos. Data significant divided into three significant subgroups where subgroup 1 (5 and 6 weeks), significant different then subgroup 2 (4th week) and then those of subgroup 3 (7th week) using Duncan Multiples range test for comparative of means.

Table (1)Epidemiological sheet of the investigated chicken farms for IBV- infection.

| Serial No. | Governorate | Chicken type | Breed | Age | House Capacity | Housing System | Vaccination against IB | Problem | |
|------------|---------------|--------------|-------------|------|----------------|----------------|------------------------|--|--------------------------------|
| | | | | | | | | Signs | PM |
| (1) | Giza | Layer | Lohman | 16.w | 14.000 | Cages | Yes (L) | Resp + Ent | Resp + Ent |
| (2) | Menofia | Broiler | Arbor-Acres | 32.d | 5100 | Deep litter | Yes (L) | Resp | Resp |
| (3) | Giza | Layer | Lohman | 16.w | 7.000 | Cages | Yes (L) | Resp + Ent | Resp + Ent |
| (4) | Kalubia | Broiler | Hubbard | 39.d | 8.000 | Deep litter | Yes (L) | Resp | Resp |
| (5) | Kafr-ElShikh | Broiler | Arbor-Acres | 36.d | 4.800 | Deep litter | No | Resp + Ent | Resp + Ent |
| (6) | Giza | Broiler | Hubbard | 41.d | 6000 | Deep litter | Yes(L) | Resp + Ent | Resp + Ent |
| (7) | Dakahlia | Breeder | Cobb | 33.w | 7100 | Deep litter | Yes (L +I) | Egg drop (30%) +↓ fert + ↓hatch + deformity | Myopath + Ren + peritonitis |
| (8) | Kafr-ElShikh | Broiler | Arbor-Acres | 34.d | 4800 | Deep litter | No | Resp | Resp |
| (9) | Fayoum | Broiler | Avian | 32.d | 3600 | Deep litter | No | Resp | Resp + Ren |
| (10) | Kalubia | Broiler | Arbor-Acres | 34.d | 3400 | Deep litter | No | Resp | Resp |
| (11) | Sharkia | Broiler | Hubbard | 34.d | 7200 | Deep litter | No | Resp | Resp + Ren |
| (12) | Sharkia | Layer | ISA | 18.w | NR | Cages | Yes (L + I) | Resp + Ent + Mort | Resp + Ent |
| (13) | Menofia | Broiler | Hubbard | 25.d | 5300 | Deep litter | No | Resp + Ent | Resp + Ent + Ren |
| (14) | Suez | Broiler | Hubbard | 25.d | 6770 | Deep litter | No | Resp + Ent | Resp + Ren |
| (15) | Kafr-ElShikh | Broiler | Hubbard | 40.d | 4000 | Deep litter | No | Resp | Resp + Ent |
| (16) | Dakahlia | Broiler | Hubbard | 24.d | 6200 | Deep litter | Yes (L) | Resp + Ren | genital |
| (17) | Behira | Breeder | Cobb | 25.w | 20.000 | Deep litter | Yes (L + I) | Egg drop (8%) + egg deformity | Resp |
| (18) | Dakahlia | Broiler | Hubbard | 32.d | 5600 | Deep litter | Yes (L) | Resp + Mort (7%) | Resp + Ent |
| (19) | Dakahlia | Broiler | Hubbard | 45.d | 7200 | Deep litter | No | Resp + Ent | Resp + Ent + Ren |
| (20) | Giza | Breeder | Arbor-Acres | 69.d | 3000 | Deep litter | Yes (L) | Resp + Ent | Resp + Ent |
| (21) | Suez | Broiler | Hubbard | 39.d | 8000 | Deep litter | Yes (L) | Resp + Ent | Resp + Ent |
| (22) | Giza | Broiler | Avian | 39.d | 6000 | Deep litter | Yes (L) | Resp + Ent | genital |
| (23) | Giza | Layer | Lohman | 41.w | 14.000 | Cages | Yes (L + I) | Egg drop (30%) | genital |
| (24) | Gharbia | Layer | Lohman | 54.w | 14.000 | Cages | Yes (L + I) | Egg drop (14%) + egg deformity | genital |
| (25) | Dakahlia | Breeder | Aror-Acres | 34.w | 6000 | Deep litter | Yes (L + I) | Egg drop + egg deformity | |
| (26) | GIZA | Broiler | Arbor-Acres | 42.d | 45600 | Deep litter | No | Resp. | Resp. |
| (27) | Kafr-El Shikh | Broiler | Arbor-Acres | 42.d | 5100 | Deep litter | No | Resp. | Resp. |
| (28) | GIZA | Broiler | Arbor-Acres | 26.d | 4000 | Deep litter | No | Resp. | Resp. |
| (29) | Kalubia | Broiler | Cobb | 37.d | 3500 | Deep litter | No | Resp. + Ent. | Resp + Ent |
| (30) | Sharkia | Broiler | Hubbard | 26.d | 7000 | Deep litter | Yes (L) | Resp. | Resp. |
| (31) | Ismalia | Broiler | Hubbard | 43.d | 4500 | Deep litter | No | Resp. | Resp + Ren. |
| (32) | Behera | Broiler | Baladi | 25.d | 3500 | Deep litter | No | Resp. | Resp. |
| (33) | Domiate | Broiler | Hubbard | 31.d | 4800 | Deep litter | Yes (L) | Resp. + Ent. | Resp + Ent |
| (34) | Alexandria | Layer | Bovans | 49.w | 20.000 | Cages | Yes (L + I) | Egg drop 18% | genital |
| (35) | GIZA | Breeder | Hubbard | 26.w | 20.000 | Deep litter | Yes (L + I) | Delay production | genital |
| (36) | Dakahlia | Breeder | Hubbard | 34.w | 9.000 | Deep litter | Yes (L + I) | Egg drop 8% | genital |

L = Live vaccine.

Resp = Respiratory I = Inactivated vaccine.

Ent = Enteric

↓ fert = reduce fertility. Myopath = Myopathy ↓ hatch = reduce hatchability

Mort=Mortality

NR : Not recorded.d = day

w = week

Table (2):Collective sheet of the total 36 investigated chicken farms.

| <i>Item</i> | | <i>No</i> | <i>History of vaccination</i> | | | |
|----------------------|-------------------------------|-----------|-------------------------------|----------|----------------------|----------|
| | | | <i>Vaccinated</i> | | <i>Nonvaccinated</i> | |
| | | | <i>No</i> | <i>%</i> | <i>No</i> | <i>%</i> |
| Governorates | | 13 | | | | |
| Total examined farms | | 36 | 21 | 58.3 | 15 | 41.6 |
| Bird type, | Broiler | 24 | 9 | 37.5 | 15 | 62.5 |
| | Layer (total) | 6 | 6 | 100 | 0.0 | 0.0 |
| | Layer (replacement) | 3 | 3 | 100 | 0.0 | 0.0 |
| | Layer (laying) | 3 | 3 | 100 | 0.0 | 0.0 |
| | Broiler breeder (total) | 6 | 6 | 100 | 0.0 | 0.0 |
| | Broiler breeder (replacement) | 1 | 1 | 100 | 0.0 | 0.0 |
| | Broiler breeder (laying) | 5 | 5 | 100 | 0.0 | 0.0 |

**Field cases of natural infection with IB in broiler chickens.**

Fig. (1 A & B): chicken showing respiratory signs (congested eye and nasal discharge) and watery feces with soiled vent.

Fig. (2): Chicken mucosa of trachea with severe congestion.

Fig. (3): Congested lung, air sacculitis, nephritis and deposition of uric acid in ureter.

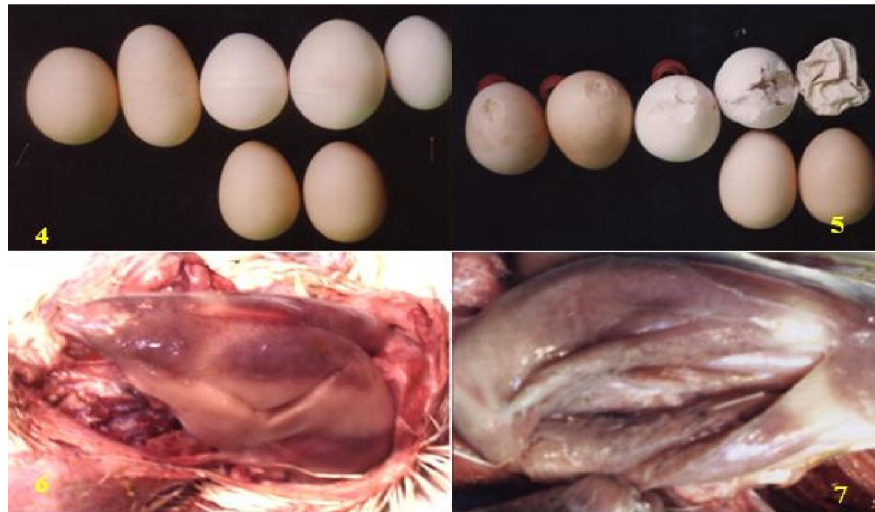


Fig. (4): Abnormality of egg shape (misshapen and pale color) in naturally infected broiler breeder farm with IB.
Fig. (5): Abnormality of egg shell showing variable soft shell in naturally infected broiler breeder farm with IB.
Fig. (6): Field case of natural infection with IB in broiler breeder hen, had superficial pectoral myopathy.
Fig. (7): Field case of natural infection with IB in broiler breeder hen, showing deep pectoral myopathy.

Table (3):Collective results of isolation and identification of IBV by egg inoculation.

| Type | Total No. examined farms | No. of Farms positive for IBV isolation | % |
|---------|--------------------------|---|-------|
| Broiler | 24 | 16 | 66.66 |
| Layer | 6 | 5 | 83.3 |
| Breeder | 6 | 4 | 66.66 |

Table (4): Incidence of IBV infection in 16 positive broiler chicken farms in relation to age.

| Farm | Ag/weeks | | | |
|---|----------|------|------|------|
| | 4 | 5 | 6 | 7 |
| Number of postive broiler farms for IBV isolation | 3 | 6 | 6 | 1 |
| Incidence % | 18.75 | 37.5 | 37.5 | 6.25 |

*** Statistical analysis**

| Farm | Ag/weeks | | | |
|---------------------|----------|------|------|------|
| | 4 | 5 | 6 | 7 |
| Sub group 1 | | 37.5 | 37.5 | |
| Subgroup 2 | 18.75 | | | |
| Subgroup 3 | | | | 6.25 |
| Fischer exact value | 26.5418* | | | |

- Significant at $p < 0.05$ using Fischer Exact probability test for comparative of means.

Table (5): Percentage of Embryonic lethality followign IBV isolates inoculation of 9-10 days SPF egg embryos during 5 passages.

| <i>Isolate Code</i> | <i>Chicken type</i> | <i>Breed</i> | <i>Age</i> | <i>Vaccination against IB</i> | <i>Embryo lethality %/passages</i> | | | | |
|---------------------|---------------------|---------------|------------|-------------------------------|------------------------------------|------------|------------|------------|------------|
| | | | | | <i>p.1</i> | <i>p.2</i> | <i>p.3</i> | <i>p.4</i> | <i>p.5</i> |
| 1 | Layer | Lohman | 16.w | Yes (L) | 0 | 0 | 0 | 12.7 | 62.5 |
| 2 | Broiler | Arbor-Acres | 32.d | Yes (L) | 0 | 0 | 50 | 50 | 62.5 |
| 3 | Layer | Lohman | 16.w | Yes (L) | 0 | 25 | 0 | 37.5 | 71.5 |
| 4 | Broiler | Hubbard | 39.d | Yes (L) | 0 | 0 | 40 | 71.5 | 100 |
| 5 | Broiler | Arbor-Acres | 36.d | No | 0 | 0 | 0 | 28.5 | 57 |
| 6 | Broiler | Hubbard | 41.d | Yes (L) | 20 | 25 | 0 | 16.6 | 50 |
| 7 | Breeder | Cobb | 33.w | Yes (L + I) | 20 | 0 | 0 | 62.5 | 100 |
| 8 | Broiler | Arbor - Acres | 34.d | No | 0 | 20 | 25 | 0 | 0 |
| 9 | Broiler | Avian | 32.d | No | 0 | 20 | 40 | 100 | 100 |
| 10 | Broiler | Arbor-Acres | 34.d | No | 25 | 0 | 0 | 28.5 | 71.4 |
| 11 | Broiler | Hubbard | 34.d | No | 0 | 100 | 100 | 100 | 100 |
| 12 | Layer | ISA | 18.w | Yes (L + I) | 0 | 0 | 0 | 0 | 57 |
| 13 | Broiler | Hubbard | 25.d | No | 0 | 0 | 20 | 66.6 | 100 |
| 14 | Broiler | Hubbard | 25.d | No | 0 | 25 | 50 | 100 | 100 |
| 15 | Broiler | Hubbard | 40.d | No | 0 | 80 | 60 | 62.5 | 43 |
| 16 | Broiler | Hubbard | 24.d | Yes (L) | 20 | 75 | 0 | 50 | 0 |
| 17 | Breeder | Cobb | 25.w | Yes (L + I) | 0 | 0 | 50 | 43 | 100 |
| 18 | Broiler | Hubbard | 32.d | Yes (L) | 0 | 20 | 66.6 | 57 | 83 |
| 19 | Broiler | Hubbard | 45.d | No | 0 | 40 | 0 | 25 | 43 |
| 20 | Breeder | Arbor-Acres | 69.d | Yes (L) | 20 | 0 | 25 | 50 | 100 |
| 21 | Broiler | Hubbard | 39.d | Yes (L) | 0 | 0 | 20 | 37.5 | 12.5 |
| 22 | Broiler | Avian | 39.d | Yes (L) | 0 | 0 | 25 | 100 | 100 |
| 23 | Layer | Lohman | 41.w | Yes (L + I) | 0 | 0 | 50 | 71.4 | 100 |
| 24 | Layer | Lohman | 54.w | Yes (L + I) | 0 | 60 | 100 | 100 | 100 |
| 25 | Breeder | Arbor-Acres | 34.w | Yes (L + I) | 25 | 0 | 0 | 71.4 | 71.4 |

W = week d = day L = Live Vaccine I = Inactivated vaccine

Dead embryos within 24 hours post inoculation were discarded from calculation.

Table (6): Collective mean percentage of embryonic lethality following 25-IBV inoculation of 9-10 days SPF egg embryos during 5 passages.

| <i>IBV isolate numbers</i> | <i>Embryonic lethality %/ Passages</i> | | | | |
|----------------------------|--|------------|------------|------------|------------|
| | <i>p.1</i> | <i>p.2</i> | <i>p.3</i> | <i>p.4</i> | <i>p.5</i> |
| 25 | 5.2 | 19.6 | 28.86 | 53.68 | 71.39 |

p = passage level

Table (7):Collective results of embryonic weight reudction % of survived embryos for 25 IBV isolates at the level of fourth and fifth passage.

| Isolate Code | Embryo weight reduction % | | Dwarfing | Isolate Code | Embryonic weight reduction % | | Dwarfing |
|--------------|---------------------------|------|----------|--------------|------------------------------|--------------|----------|
| | p.4 | p.5 | | | p.4 | p.5 | |
| 1 | 9.2 | 19.2 | Neg. | 14 | 5.7 | 7.3 | Neg. |
| 2 | 15.5 | 18.3 | Neg. | 15 | 6.9 | 8.2 | Neg. |
| 3 | 5.3 | 19.5 | Neg. | 16 | NR | 56.6 | Post. |
| 4 | 17 | 24.5 | Post. | 17 | 22.5 | 39.3 | Post. |
| 5 | 16.04 | 24.5 | Post. | 18 | 13.2 | 21.4 | Neg. |
| 6 | 9.2 | 13.1 | Neg. | 19 | 19.3 | 22.8 | Neg. |
| 7 | 15.8 | 29.7 | Post. | 20 | 10.2 | 9.5 | Neg. |
| 8 | 15.6 | 21.3 | Neg. | 21 | 14.3 | 20.6 | Neg. |
| 9 | 23.6 | 30.8 | Post. | 22 | 5.2 | 12.9 | Neg. |
| 10 | 7.6 | 17.6 | Neg. | 23 | 24.0 | 25.6 | Post. |
| 11 | 23.8 | 30.4 | Post. | 24 | 28 | 35.9 | Post. |
| 12 | 11.8 | 14.3 | Neg. | 25 | 7.6 | 17.6 | Neg. |
| 13 | 8.6 | 3.8 | Neg. | Mean | 13.99 ± 1.38 | 21.78 ± 2.32 | |

- p = passage level. Mean = mean ± S.E. Post.= Positive Neg.=Negative
- Embryo weight reduction% = A weight differential of 25 percent or more between infected and normal embryos of the same age may be considered evidence of viral infection, (Anon, 1963).
- Embryonic reduction % = $\frac{\text{Mean weight of inoculated embryos}}{\text{Mean weight of un inoculated}} \times 100$

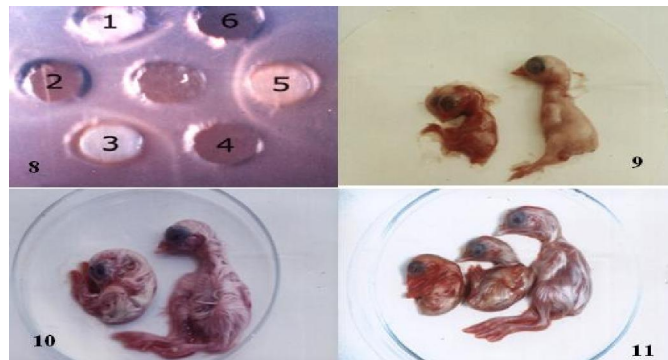


Fig. (8): Laboratory identification of field isolates of IBV using AGPT. Central well contain positive precipitating serum against IBV, wells 2, 4, 6 are empty wells 1 and 3 contain CAM homogenates of tested samples, and well 5 contain positive standard precipitating IBV antigen. Precipitating lines obtained with tested (1 and 3) and reference (5) antigens.

Fig. (9): Lethal IBV showing stunted SPF chick embryo 72 h pi at the level of the 4th embryonic passage (left) as compared with non infected control (right) of the same age.

Fig. (10): Non lethal IBV showing SPF chick embryo with stunted, hemorrhagic, feet deformity at 18 days of age (left) as compared with non infected control (right) of the same age.

Fig. (11): Non lethal IBV showing SPF embryo with curling, stunting, hemorrhages and feet deformity at 18 days of age at the level of the fourth embryonic passage (left) as compared with non infected control (right) of the same age.

2.b The influence of different strains IB virus on chicken embryo kidney cells (CEK).

(Table 8), Twenty IBV isolates originated from choriollantoic fluids harvested from the 5th egg embryo passage were used as inoculum. Focal cytopathic effect (CPE) started to observe 24-48 hours pi (under inverted microscope in unstained culture), followed by extensive CPE on the 3rd day pi, but were

seen in later passages after 24 hours incubation. These gradual changes are described as follows: 1. Foci of refractile round cells and occasional syncytia (Fig.12). 2. The affected cells became detached from the monolayer and tended to aggregate in clumps that floated free in the nutrient medium. 3. Appearance of porous large area distinctly demarcated from the rest of the cells. (Fig.13-17).

Table (8): Results of inoculation of IBV isolates in chicken embryo kidney (CEK) cells.

| <i>Isolate Code</i> | <i>Cytopathic effect*/Passage No.</i> | | | |
|---------------------|---------------------------------------|------------------|------------------|------------------|
| | <i>Passage-1</i> | <i>Passage-2</i> | <i>Passage 3</i> | <i>Passage 4</i> |
| 1 | + | + | ++ | +++ |
| 2 | (-) | + | ++ | +++ |
| 3 | + | ++ | +++ | +++ |
| 4 | + | ++ | +++ | +++ |
| 5 | (-) | + | ++ | +++ |
| 6 | + | + | ++ | +++ |
| 7 | NT | NT | NT | NT |
| 8 | + | ++ | +++ | +++ |
| 9 | (-) | + | ++ | +++ |
| 10 | (-) | + | ++ | +++ |
| 11 | + | ++ | +++ | +++ |
| 12 | NT | NT | NT | NT |
| 13 | (-) | + | ++ | +++ |
| 14 | NT | NT | NT | NT |
| 15 | + | ++ | ++ | +++ |
| 16 | + | ++ | ++ | +++ |
| 17 | + | + | ++ | +++ |
| 18 | + | ++ | +++ | +++ |
| 19 | (-) | + | ++ | +++ |
| 20 | NT | NT | NT | NT |
| 21 | + | ++ | +++ | +++ |
| 22 | NT | NT | NT | NT |
| 23 | + | +++ | +++ | +++ |
| 24 | + | ++ | +++ | +++ |
| 25 | + | ++ | ++ | +++ |

NT = not tested.

* inverted microscopy examined in unstained culture.

+ = Focal involvement.

++ = partial involvement.

+++ = Extensive involvement.

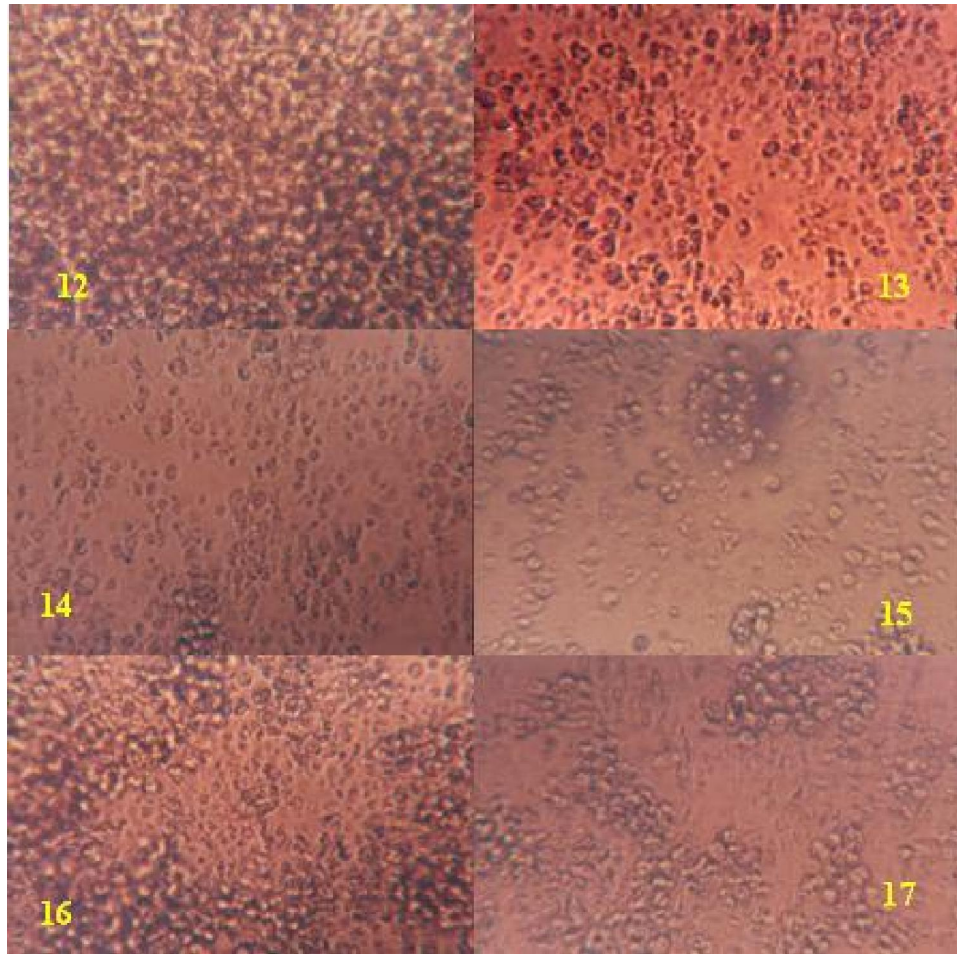
Table (9): Collective results “incidence percentage” of CPE in CEK-cell culture.

| <i>No. of examined samples</i> | <i>No. and percentage of CPE</i> | | | | | | | |
|--------------------------------|----------------------------------|----------|---------------|----------|---------------|----------|---------------|----------|
| | <i>Pass.1</i> | | <i>Pass.2</i> | | <i>Pass.3</i> | | <i>Pass.4</i> | |
| | <i>Post.</i> | <i>%</i> | <i>Post.</i> | <i>%</i> | <i>Post.</i> | <i>%</i> | <i>Post.</i> | <i>%</i> |
| 20 | 14 | 70 | 20 | 100 | 20 | 100 | 20 | 100 |

Post.= Positive

.No.= Number.

Pass.= Passage.



- Fig. (12):** Control non-infected monolayer of CEKC, 48 hr after culturing.
- Fig. (13):** Characteristic CPE produced by IBV at passage level-2. Affected cells detached from monolayer and tend to aggregate in clumps (unstained culture).
- Fig. (14):** Characteristic CPE produced by IBV at passage level-3. Increased areas of detached cells and tend to aggregate in clumps (unstained culture).
- Fig. (15):** Characteristic CPE produced by IBV at passage level-2. Affected cells became refractile rounded (unstained culture).
- Fig. (16):** Characteristic CPE produced by IBV at passage level-3. Porous large area distinctly demarcated from the rest of cells (unstained culture).
- Fig. (17):** Characteristic CPE produced by IBV. Isolated areas of detached cells fuse together and cause coalescent areas of detached cells (unstained culture).

4. Discussion

Infectious bronchitis (IB) virus, first described in 1930 (Schalk and Hawn, 1931), continues to be a major cause of disease in chickens of all ages and types in all parts of the world (Anon, 1988, 1991). The disease is prevalent in all countries with an intensive poultry industry, with the incidence of infection approaching 100% in most locations (Ignjatovic and Sapats, 2000). The disease is primarily a respiratory infection of chickens. Nevertheless, three clinical manifestations are generally observed in the field, namely: respiratory

disease, reproductive disorders and nephritis (Cavanagh and Naqi, 1997; McMartin, 1993). Concerning the prevalence of IB outbreaks in some locations in Egypt, in the present investigations, examination of 36 chicken farms distributed in 13 governorates, representing broilers, layers and broiler breeder farms revealed that the IBV is prevalent in Egypt, since the initial description and isolation of the virus (Ahmed, 1954; Eissa et al., 1963; Ahmed, 1964; Amin and Mustaggar, 1977 and

El-Kady, 1989). Occurrence of the disease in unvaccinated 9 broiler farms out of 24 examined broiler farms (37.5%), tables (1 and 2), was expected finding due to the highly contagious nature of the disease (Cavanagh and Naqi, 2003) and the method of spread is airborne or mechanical transmission between birds, houses and farms. Airborne transmission is via aerosol and occurs readily between birds kept at a distance over 1.5 meter. Prevailing winds might also contribute to spread between farms that are separated by a distance of as much as 1,200 meter (Cumming, 1970). On the otherhand, occurrence of the disease in (7) vaccinated broiler farms (29.16%) and (5) vaccinated layer farms and (4) vaccinated broiler breeder farms, was also expected, based on the presence of large number of antigenic serotypes (Cook and Huggins, 1986; Gelb et al., 1991; Gubillos et al., 1991) and emerge of new IBV variants with nephropathogenic property of most of them was the characteristic of the recent history of the disease in Egypt in the last six years by many investigators (El-Sisi and Eid, 2000; Lebda et al., 2004; Sultan et al., 2004). Also, the long life span of layers and broiler breeders is favorable for the evolution of new serotypes as well as, immune selective pressure produced by intensive live and inactivated vaccination, maintenance of multi-age flocks for continual production, periodic introduction of pullets, infrequent clean out and disinfection of the premises, and the recycling of the virus in the flocks resulting in great opportunity for infections and spreading of the disease (Gelb et al., 1991 and Gelb et al., 1997). This speculation was the main objective of the present investigations.

Recording of the respiratory form of the disease as the most observed syndrome, mostly in broiler farms beyond 4 weeks of age and to less extent in replacement layer and breeder and laying hens were similar to those described by (McMartin, 1993; Cavanagh and Nagi, 1997). Occurrence of other clinical signs and necropsy, resembled those reported by several reports including wet eyes, swollen sinuses; reduced feed consumption and body weight, varying mortality (Hofstad, 1984), wet droppings (Bumstead et al., 1989), declines in egg production, quality abnormality of eggs and hatchability (Cook et al., 1987), breeder myopathy of pectoral muscles (Parsons et al., 1992), respiratory lesions (Hofstad, 1984), renal lesions (Gough et al., 1992) and genital lesions (Hofstad, 1984), swelling of glandular stomach (Wang et al., 1998) and haemorrhagic ulceration of the glandular stomach. Conclusively, the present study confirms that the epidemiology of IB in Egyptian chicken farms in a continuous problem, and none of the countries which have an intensive poultry industry are free from IBV.

Although attempts have been made, at the regional level, to keep flocks free from IBV, but without successful results. Given the highly infectious nature of the virus, even the strictest preventative measures are sometimes not sufficient (Ignjatovic and Sapats, 2000). Under normal flock management with "all-in/all-out" operations, cleaning and disinfections between batches have limited the level of infection to a minimum, however, exclusion of IBV had not been achieved through such measures (Ignjatovic and Sapats, 2000).

Primary isolation of IBV, based on inoculation in 9-11 day old SPF (Anon, 1963; Gelb and Jackwood, 1998) was adopted which could cover three important objectives. (1) Isolation and identification of IBV. (2) Determination of virus lethality. (3) Recording embryo gross abnormality (stunting and dwarfing effect of the virus).

The tissue tropism of IBV strains seemed to be wide and variable (Lucio and Fabricant, 1990). The presence of the IBV in the respiratory and urogenital tract of chickens could be well documented. Different strains of IBV had been isolated from spleen, feces, cecal tonsils, cloacal content, semen, eggs, bursa and oesophagus as reported by (Lucio and Fabricant, 1990). Generally, it has been assumed that the cecal tonsils and kidneys could be considered an important sites for the persistence of IBV, as the virus has been recovered from these tissues for a prolonged period as also mentioned by Alexander et al., (1978) and we think that to avoid false negative results the specimens taken for IBV isolation must include trachea, lung, kidney, and cecal tonsils as also mentioned by (Jose et al., 2000). On primary isolation, gross pathological alterations of the embryo were employed as evidence of viral activity. While embryo mortality was not a constant finding on initial passage as also mentioned by (Cunningham and Jones, 1953). In some cases as many as three or four serial passages may be necessary before detection of IBV infection, based on embryo death or lesions and the serial passage of IBV in eggs was accompanied by an increase in virulence for embryos (Bijlegna, 1960; and Anon, 1963). Therefore, five passages were performed in the present study before the virus-isolation attempt was considered as negative.

Using of CAM homogenate of inoculated embryos in agar gel precipitation (AGP) test against positive reference precipitating sera gave specific positive precipitin band(s) in 25 IBV isolates (Table 3), as also correspond to the findings of Woernle (1966) and Hofstad (1981); who concluded that the AGP test was suitable and specific for identifying field isolates as IBV as it could detect group specific antigen common to all IBV strains and

serotypes. Deaths of few embryos at initial first passage (5.2%), followed by increasing to 19.6%, 28.8%, 53.6% and 71.3% on subsequent 2nd, 3rd, 4th and 5th passages tables (4 and 5), accompanied by embryos dwarfing which was more evidence at level of 5th passage (9 out of 25 isolates “36%”) (Table 6 & 7). These findings was explained as that the serial passages of IBV in egg embryos was accompanied by an increase in virulence for embryos (Anon, 1963; Cavanagh and Naqi, 2003), although some IBV isolates did not cause dwarfing of the inoculated embryos after serial passage (Clark et al., 1972). Among the alterations which were considered most typical of IBV infection were weak living embryos, curling of embryos with feet deformed compressed over the head, and presence of urates in the persistent mesonephron were also reported by (Anon 1963; OIE, 1996; Cavanagh and Naqi, 1997).

For primary isolation of IBV, chicken kidney cell culture was not recommended, because the virus required adaptation in embryonating eggs before it will grow in cell culture as recommended by (Gelb and Jackwood, 1998; Cavanagh and Naqi, 2003). For this, 20 IBV previously adapted to propagate in embryonated eggs up to five passages were used as inoculum in chicken embryo kidney (CEK) cell, for four blind passages. Results of tables 8 & 9 and Figs. 12-17, revealed that all the isolated IBV, were adapted and grew in CEK cell culture. Six isolates (30%) did not induce characteristic CPE in the first passage, while the other 16 isolates (70%) could induce characteristic CPE. By re passage of 20 examined isolates in CEK cells all were successfully adapted and CPE developed at 100%, 100%, 100% at the levels 2nd, 3rd and 4th passage; respectively. Cytopathic changes produced by the IBV strains were granularity and vacuolization of the cytoplasm. The affected cells became detached from the monolayer and tend to aggregate in clumps that floated free in the nutrient medium. Appearance of porous large area distinctly demarcated from the rest of the cells. Multinucleated giant cells (syncytia) were not numerous in early passages, but were seen in later passages of all strains when examined after 24 hours incubation, these findings were similar to those reported previously (Hopkins, 1974).

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