

## The Prevalence of Infectious Bronchitis (IB) Outbreaks in Some Chicken Farms. II. Molecular Characterisation of Field Isolates IB Virus.

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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. The viruses were isolated and identified previously by chicken embryo, CEK cell culture inoculation and now by RT-PCR applied on RNA of IBVs. Four variants isolates of IBV were choised from the previous field isolates from three broiler farms and one from layer farm. All the examined farms were vaccinated using the commercial live IB-H<sub>120</sub> vaccine in addition to the IB-inactivated vaccine in the layer farm. Typing of the present isolates based on sequencing of a part of the S1 gene, revealed four IBVs could be aligned and matched with homology to Asia, Europe, USA and Middle East strains. Four groups of one-day-old SPF chicks were inoculated with the four variants IBV isolates at 1 day old to test the virulence of those isolates. The results at 2 weeks pi revealed that all isolates were able to induce serological resposne postinfection, respiratory distress and depression commenced at 24 hours postinfection. 20% and 100% mortality was recorded with isolates 4 and 23; respectively. Assessment of pathogenicity index and pathotyping (at the end of observation period "2wk-pi"), categorized to tested 4 isoaltes (4, 16,18, 23) into three isoaltes of high virulent (4, 18 and 23), and one isolate of intermediate virulent (16). About 50% reduction in body weight as recorded with the four IBV isolates 2 wk postinfection. Kidney lesions were grossly, nephritis-nephrosis with urate deposition in ureters, while microscopic lesions were increased the amount of rough endoplasmic reticulum (RER). Tracheal lesions were grossly increased amount of mucin, while microscopic lesions were edema of mucosa and inflammatory cells in the lamina propria. [Journal of American Science 2010;6(9):71-93]. (ISSN: 1545-1003).

**Keywords:** Infectious Bronchitis (IB); Outbreaks; Chicken Farms; Molecular Characterisation; Field Isolates

### 1. Introduction

IBV, a prototype of coronaviridae family, consists of three major structural proteins : the phosphorylated nucleocapsid (N) protein which is internally located, the membrane (M) glycoprotein , and the spike (S) glycoprotein, which is posttranslationally cleaved into N-terminal S1 (92 KD) and C-terminal S2 subunits (84 KD) as described later (Cavanagh and Naqi, 1997). The S1 subunit is hypervariable, whereas the N protein contains some regions whose predicted amino acid sequences tend to be conserved among IBV strains (Williams et al., 1992).

Serotypic evolution in IBV are associated primarily with changes in the sequence of the S1 glycoprotein, which contains regions associated with virus attachment and epitopes that induce production of neutralizing antibodies (Cavanagh and Davis, 1988; Cavanagh et al., 1988). Different serotypes, subtypes, and variants of IBV are thought to be generated by nucleotide point mutations, insertions or deletion (Kusters et al., 1987; Wang et al., 1993, 1994; Jia et al., 1995), which are responsible for the problem of outbreaks of IB disease in previously vaccinated chicken flocks.

The serotypic determinants had been identified in a 395-amino acid region of the S1 subunit, which contain four variable regions. Among European IBV strains of the Mass serotype, two hypervariable regions (HVRs), HVR-1 residues (56-69) and HVR-2 (residues 117-131) were identified (Kusters et al., 1987; Cavanagh et al., 1988). Isolates from the United States had similar HVRs in the S1 subunit between residues (53-148) (Wang et al., 1993). The virus neutralization (VN) antibodies that form the basis for comparison of IBV isolates were induced largely by the N-terminus of the S1 protein (Koch et al., 1990). The 120 residues of the N protein C-terminal contain cytotoxic T lymphocytes (CTL) epitopes that may decrease viral load and induce protective immunity by inducing CTL response in the chicken (Seo and Collisson, 1997).

In Egypt, IB was first described by Ahmed (1954), subsequently several reports emphasized the prevalence of the disease . 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. Field isolates of IBV: were obtained previously from 36 chicken farms showing symptoms suspected to be IBV infection.

2. Serum Serum Samples: were separated and checked by Synbiotic ELISA for detection of specific IBV antibodies.

3. Experimental Chickens:  
Sufficient one-day-old chicks were hatched from SPF fertile chicken eggs obtained from (Nile SPF), incubated and hatched, floor reared under strict hygienic condition in isolated experimental rooms, previously cleaned and disinfected. Chicks were provided with commercial broiler ration, water and

feed were provided adlibidum. They used for pathogenicity test.

4. Reagents and buffers for extraction of viral RNA of IBV isolates by QIAamp® Viral RNA Mini Spin Column Kit:

IBV Isolates, Ethanol (96-100% purity) (Analar), QIAamp® Viral RNA Mini kit buffers, AVL Buffer (QIAGEN), Carrier RNA Pellet (QIAGEN), RNase free water (QIAGEN) Buffer AVL/ Carrier RNA solution (QIAGEN), AW1 Buffer, (QIAGEN), AW2 Buffer (QIAGEN), AVE Buffer (QIAGEN) and QIAamp Spine Columns.

Reagents and buffers for RT-PCR utilizing Single Tube Reaction:

IBV RNA Extract (Viral RNA extracted from IBV isolates using QIAamp viral RNA Mini Kit, was utilized in first strand synthesis cDNA.), RNase Free Water (Sigma) and Tris-EDTA Buffer (TE), PH8.0. Oligonucleotide Primers for amplification of untranslated (UTR) region of IBV isolates (QIAGEN) were designed according to Adzhar et al., (1996)). The primers were designed to amplify specific sequence in the untranslated region of genome Table (1).

**Table (1): The oligonucleotide for amplification Untranslated region (UTR) of RNA of IBV by RT-PCR assay.**

Oligonucleotide <sup>a</sup>	Sequence (5`-3`)	Gene	Location <sup>b</sup>	Location reference
UTR2+	AAGGAAGATAGGCATGTAGCTT	3` UTR	234-255	Williams et al., (1993)
UTR1-	GCTCTAACTCTATACTAGCCTAT	3` UTR	509-531	Williams et al., (1993)

<sup>a</sup>+ = genome sense; - = antigenome sense

<sup>b</sup> The numbers correspond to the nucleotide positions in the indicated references.

Oligonucleotide Primers for amplification of part of S1 gene of IBV isolates by RT-PCR (QIAGEN) were designed according to Cavanagh et al.,( 1999).

The primers were designed to detect and differentiate three types of IBV: 793/B (also known as 4/91 and CR88), Massachusetts and D274, using the S1 region of the S protein gene. Table (2).

QIAGEN One- Step Enzyme Mix, HotStar Taq® DNA Polymerase, One Step RT-PCR Buffer (5xconcentrate) and Deoxynucleotide Triphosphates (dNTPs).

5. Detection of IBV RNA by Polymerase Chain Reaction (PCR) according to Gelb et al., (2005).

**Table (2): The oligonucleotide for amplification of S1 region of S protein gene for three types of IBV: 793/B, Massachusetts and D274.**

Oligonucleotide	Sequence (5`-3`)	Gene	Position in sequence	Reference
XCE1+ <sup>a</sup>	CACTGGTAATTTTTTCAGATGG	S1	728 to 749	Adzhar et al., (1997)
XCE3- <sup>b</sup>	CAGATTGCTTACAACCACC	S1	1093 to 1111	Adzhar et al., (1997)

<sup>a, b</sup> Positive and negative sense oligonucleotides, respectively that include :

**Table (3): Reaction components for one-step RT-PCR for amplification of UTR of RNA of IBV isolates.**

Component	Volume/ reaction
Master Mix	
RNase free water	20µl
5x QIAGEN One Step RT-PCR Buffer	10µl
dNTP Mix (containing 10mM of each dNTP)	2.0µl
Forward primer	3.0µl
Reverse primer	3.0µl
QIAGEN One Step RT-PCR Enzyme Mix	2.0µl
	40µl

**Table (4): The cycling protocol used in RT assay during amplification of UTR of IBV genome:**

Protocol steps	Temperature	Time	No. of Cycles
Reverse transcription	50°C	30mint	1 cycle
Initial PCR activation step	95°C	15mint	1 cycle
Denaturation	95°C	1min	} 35 cycle
Primer Annealing	56°C	1min	
Polymerization	72°C	2 min	
Final extraction	72°C	7 min	1 cycle
Cooling	15°C	Indefinite hold	

6. Buffers and reagents for agarose gel electrophoresis: 1.0% Agarose (Hispanagar), Ethidium Bromide (Sigma), Loading buffer, and Tris Acetate EDTA Buffer (TAE) according to Adzhar et al., (1996). PCR Marker (DNA ladder) (AB gene). BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystem catalog No. 4337450) include: Ready Reaction Mix, pGEM® -3zf (+) double-stranded DNA Control Template, -21M13 Control Primer (forward), BigDye Terminator v1.1

Sequencing Buffer (5x), DyeEx 2.0® Spin kit (QIAGEN catalog No. 63206) and Dye Ex 2.0 Spin column.

7. Detection IBV and sequencing of a piece of the S1 Gene according to *Gelb et al., (2005)* Scoring indexes for clinical, lesions and pathogenicity were recorded according to Avellaneda et al., (1994); Wang and Huang, (2000) as follows:

**Table (5): Reaction components for one-step RT-PCR for amplification of part of S1 gene of IBV isolates.**

Component	Volume/ reaction
Master Mix	
RNase free water	31.0µl
5x QIAGEN One Step RT-PCR Buffer	10.0µl
dNTP Mix (containing 10mM of each dNTP)	2.0µl
Forward primer	1.5µl
Reverse primer	1.5µl
QIAGEN One Step RT-PCR Enzyme Mix	2.0µl
	48µl

**Table (6)The cycling protocol used in RT assay during part of S1 gene amplification**

Protocol steps	Temperature	Time	No. of Cycles
Reverse transcription	50°C	30mint	1 cycle
Initial PCR activation step	95°C	15mint	1 cycle

Denaturation	95°C	30 Sec	} 35 cycle
Primer Annealing	50°C	30 Sec	
Polymerization	72°C	1 min	
Final extraction	72°C	10 min	1 cycle
Cooling	15°C	Indefinite hold	

**Table (7): The cycling protocol used in sequence PCR of a part of the S1 gene of IBV isolates.**

Temperature	Time	No. of Cycle
94°C	10 Sec	} 25 Cycle
50°C	5 Sec	
60°C	2 min	
20°C	Hold until ready to purify	

**a) Clinical signs score system of infected chicks**

Clinical signs	Score
No clinical signs	0
Lacrimation, slight head shaking and watery faces	1
Lacrimation, presence of nasal exudate, depression, watery faces	2
Strong (lacrimation, presence of nasal exudate, depression), sever watery faces.	3

**b) Gross lesion scores (trachea and kidney) system of infected chicks**

<i>Organ</i>	<i>Lesion</i>	<i>Score</i>
Trachea	No lesion	0
	Slight increase of mucin	1
	Large increase of mucin	2
	Large increase of mucin and mucosal congestion	3
Kidney	No lesion	0
	Swelling, urate visible only under steriomicroscope	1
	Swelling with visible urate	2
	Swelling with large amount of urate deposit in kidney	3

**c) Pathogenicity index: Based on formula of Wang and Huang, (2000).**

Pathogenicity group	Pathogenicity index value
Low	1-9
Intermediate	10-18
High	19

8. Enzyme-linked Immunosorbent Assay (ELISA) kits.

9. Reagents for histopathology according to (Bancroft and Steven, 1977).

**3. Results**

For confirmation of IBV isolation by detection of infectious bronchitis virus RNA in allantoic fluids harvested from inoculated eggs. The test gives a qualitative evidence for the presence or absences of IBV through three steps RNA isolation, RT-PCR,

**Results** revealed that The RNA from reference and 25 IBV-field isolates was reverse-transcribed to cDNA and amplified. Examination of the amplified PCR products following electrophoresis in 1.0% agarose gel indicated that all amplified cDNA showed almost identical motilities where 298 of size product of fluorescent bands were seen as compared with the place of marker bands in the gel. That mean, all the 25 examined samples contain IBV-RNA. (Fig.1) and (table 8). The negative controls produced no PCR products.

Detection of IBV and sequencing of a piece of the S1 gene: in allantoic fluid samples by reverse transcriptase polymerase chain reaction (RT-PCR). Then a part of the S1 gene is sequenced to type the IBV strain.

Results revealed that a part of the S1 gene was yielded at 385 bp of size product of fluorescent bands were seen as compared with the place of the marker bands in the gel in four IBV isolates (Fig.2) and (table8).

1. Sequence homology of four IBV strains were about:

a. For broiler isolate code (4): Match for 100% with isolate CK/CH/LDL/011 isolated in China and Match for 100% isolate Q1 isolated in Singapore.

b. For broiler isolate code (16) Match for 98% Connecticut strain, Match for 97% M41 strain., Match for 96% with isolate Egypt /F/03 isolated in Egypt and Match for 96% with isolate GX1-98 isolated in China.

c. For broiler isolate code (18) Match for 98% with isolate 720/99 isolated in Israel and Match for 98% with isolate 885 isolated in Israel.

d. For layer isolate coded (23): Match for 93% with strain 4/91, Match for 98% with isolate FR-94047-94 isolated in France and Match for 96% with isolate Spain/92/51 isolated in Spain (table9) and (Fig.2-6).

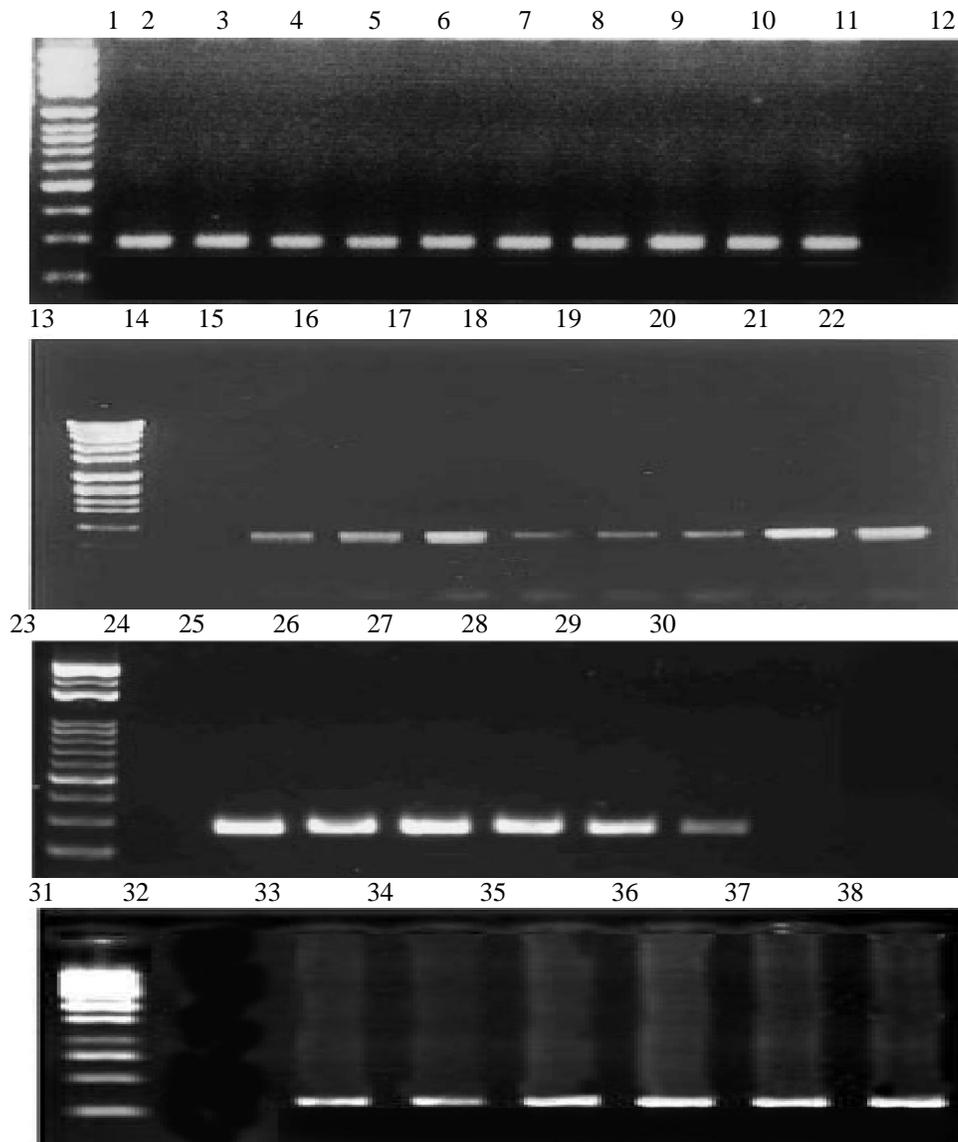


Fig. (1): RT-PCR for 25 IBV-tested AAF of egg embryos

Lanes 1, 13, 23, 31 marker.  
 Lanes 12, 14, 24, 32 Negative Control.  
 Lanes 11, 22, 30, 37, 38 positive control.  
 Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, 20, 21, 25, 26, 27, 28, 29, 33, 34, 35 and 36 field IBV tested samples with 298 bp-PCR- products.

**Table (8): Results of 25 IBV antigen assays.**

Isolate Code No.	IBV-sample reference			AGPT-CAM	RT-PCR IBV	RT-PCR IBV Typing
	Chicken Type	Age	Vaccination against IB			
1	Layer	16.w	Yes (L)	post	post	neg.
2	Broiler	32.d	Yes (L)	post	post	neg.
3	Layer	16.w	Yes (L)	post	post	neg.
4	Broiler	39.d	Yes (L)	post	post	post.
5	Broiler	36.d	No	post	post	neg.
6	Broiler	41.d	Yes (L)	post	post	neg.
7	Breeder	33.w	Yes (L+I)	post	post	neg.
8	Broiler	34.d	No	post	post	neg.
9	Broiler	34.d	No	post	post	neg.
10	Broiler	34.d	No	post	post	neg.
11	Broiler	34.d	No	post	post	neg.
12	Layer	18.w	Yes (L+I)	post	post	neg.
13	Broiler	25.d	No	post	post	neg.
14	Broiler	25.d	No	post	post	neg.
15	Broiler	40.d	No	post	post	neg.
16	Broiler	24.d	Yes (L)	post	post	post.
17	Breeder	29.w	Yes (L+I)	post	post	neg.
18	Broiler	32.d	Yes (L)	post	post	post.
19	Broiler	45.d	No	post	post	neg.
20	Breeder	69.d	Yes (L)	post	post	neg.
21	Broiler	39.d	Yes (L)	post	post	neg.
22	Broiler	39.d	Yes (L)	post	post	neg.
23	Layer	54.w	Yes (L+I)	post	post	post.
24	Layer	41.w	Yes (L+I)	post	post	neg.
25	Breeder	34.w	Yes (L+I)	post	post	neg.

Post= Postive      neg=Negative      w=Week      d=Day

**Remark:** PCR-IBV typing is done by sequencing a part of the S1 gene.

**L=Live**

**I=Inacti**

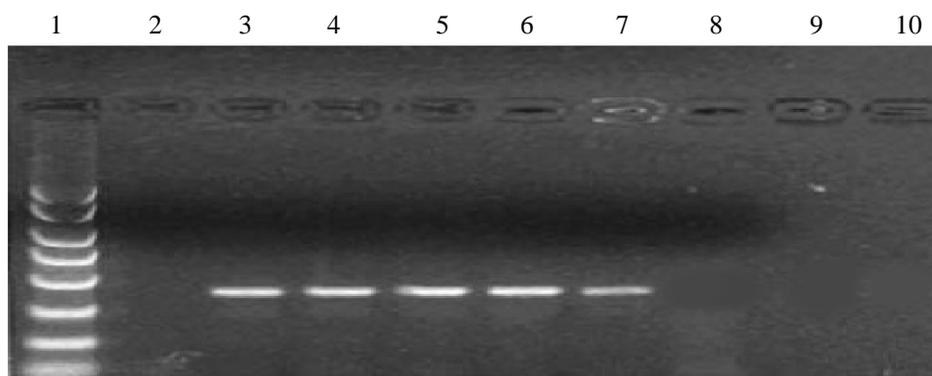
**Table (9): Results of genotyping (matching of a part of S1 gene) of four IBV field isolates.**

IB isolate code	Sample reference	Vaccination against IB	Match with data base programme (Check test file)	Match with NCBI	
				Description	Accession No.
4	Broiler, 39d	Yes (L)	No match with known type	Match for 100% with isolate CK/CH/LDL/011 isolated in China Match for 100% with isolate Q1 isolated in Singapore.	DQ167130 AF286302
16	Broiler, 24d	Yes (L)	Match for 98% with Connecticut strain. Match for 97% with	Match for 96% with isolate Egypt/F/03. Match for 96% with isolate GX1-	DQ487085 A319302

			M41 strain.	98 isolated in China.	
18	Broiler, 32d	Yes (L)	No match with known type.	Match for 98% with isolate 720/99 isolated in Israel. Match for 98% with isolate 885 isolated in Israel.	AY091552 AY279533
23	Layer, 54w	Yes (L+I)	Match for 93% with 4/91.	Match for 98% with isolate FR-94047-94 isolated in France. Match for 96% with isolate Spain/92/51 isolated in Spain.	AJ618987 DQ064801

d = day, W = week, L = Live vaccine, I = inactivated vaccine.

NCBI = National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).



**Fig. (2):** Sequencing of a part of S1 gene.

Lane 1 marker.

Lane 2 negative control.

Lanes 3, 4, 5, 6 isolates code 4, 16, 18 and 23 gave positive results at 385 bp PCR product. And Lane 7 positive control.

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GAAAGGTTTATTGTTTATAGAGAAAGTAGTGTTAACACTACCTTAGTGTT
  AACTAATTTTACTTTCTCAAATGTTAGTAACGCCCTCCTAATACAGGTG
  GTGTCATAGTATTGTTTACATCAAACACAAACAGCTCAGAGTGTTAT
  TATAATTTTAATTTCTCCTTTCTGAGTAGTTTCCGTTATGTAGAATCAGA
  TTTTATGTATGGGTCATACCACCCAAAATGTTTCATTTAGACTAGAACTA
  TTAATAATGGTTTGTGGTTTAATTCACCTTC
```

**Fig. (3):** IBV isolate (4) sequencing of 385 bp product of S1 gene. Match for 100% with isolate CK/CH/LDL/011 isolated in China and Match for 100% with isolate Q1 isolated in Singapore.

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CAGAAGTTTATTGTCTATCGTGAAAATAGTATTAATACTACTCTTAAGTT
  ACACAATTTCACTTTTCATAATGAGACTGGCGCCAACCCTAATCTTAGTG
  GTGTTTACAGAAATTTCAAACCTTACCAAACACAAACAGCTCAGAGTGTTAT
  TATAATTTTAATTTTCTCCTTTCTGAGTGGTTTTGTTTATAAGGAGTCTAA
  TTTTATGTATGGATCTTATCACCCAAGTTGTAATTTTAGACCAGAACTA
  TTAATAATGGCTTGTGGTTTAATTCACCTTC
```

**Fig. (4):** IBV isolate (16) sequencing of 385 bp product of S1 gene.

- a) Match for 98% with Connecticut strain.
- b) Match for 97% with M41 strain.
- c) Match for 96% with isolate Egypt/F/03.
- d) Match for 96% with isolate GX1-98 isolated in China.

GAAAAGTTTGTGTGTATCGTGAAAATAGTTTTAATACTACTCAGGTTTT  
 AAATAATTTACGTTTTATAATGAAAGTAATGCCCTCCTAATGTTGGTG  
 GTGTTAATACTATTAATCTTTATCAAACACATACAGCTCAGAGTGGTTAT  
 TATAATTATAATTTATCATTCTGAGTGGTTTTGTGTATAAAGCTTCTGA  
 TTTTATGTATGGATCTTATCACCCAAAGTGTGATTTTAGACCAGANACTA  
 TTAATAATGGTTTTGTGGTTTAATTCTCTATN

**Fig. (5):** IBV-isolate (18) sequencing of 385 bp product of S1 gene.

- a) Match for 98% with isolate 720/99 isolated in Israel.
- b) Match for 98% with isolate 885 isolated in Israel.

GATAGGTTTATTGTATATCGAGAAAGTAGTATTAACACTACTTTAGAGTT  
 AACTAATTTACTTTTACTAATGTAAGTAATGCTGCTCCTAACTCAGGTG  
 GCATTCAGACCTTTCAATTATATCAAACACACACCGCTCAGGATGGTTAT  
 TATAATTTAATTTATCATTCTGAGTGGGTTTTGTGTATAAACCATCTGA  
 TTTTATGTATGGGTCTTACCACCCAAAGTGTAAATTTAGACCAGAGAATA  
 TTAATAATGACTTATGGTTTAATTCATTATC

**Fig. (6):** IBV isolate (23) sequencing of 385 bp product of S1 gene.

- a) Match for 93% with 4/91.
- b) Match for 98% with isolate FR-94047-94 isolated in France.
- c) Match for 96% with isolate Spain/92/51 isolated in Spain.

2- The pathogenicity of the four IBV isolated variants by sequencing test.

isolates (coded 4, 16, 18 and 23) and divided as follow:

*Experiment design:* Seventy one-day old SPF chicks were used for evaluation of the pathogenicity of IBV

**Table(3). Experimental design of pathogenicity testing in one day old chicks.**

Group	No. of chicks	Treated groups/ isolate No.	
		Isolate Code	Inoculation dose (EID50 /ml)
1	10	* Slaughtered, serology testing	
2	10	Inoculated with isolate Code (4)	106.4
3	10	Inoculated with isolate Code (16)	106.3
4	10	Inoculated with isolate Code (18)	106.6
5	10	Inoculated with isolate Code (23)	106.0
6	10	Inoculated with live IBV vaccine (H120)	Field dose
7	10	Negative control (PBS infected)	

\*Ten serum samples were checked by ELISA at one day old (pre-experiment) to assure freedom from specific IBV antibodies

Results of ELISA revealed freedom of the tested 10 one day old chicks' sera from specific antibodies against IBV (preinfection).

1. Evidence of seroconversion : Sera collected at 14 days pi belonged to 3 IBV field isolates coded 4, 16, 18 and vaccine strain (H120) are summarised in table (4). Evidence of seroconversion are 75%, 90%, 80%, and 100% in chicks infected with IBVs 4, 16, 18, and H120; respectively. Isolate 23 not tested, where all infected chicks were dead on day 5 and 6 pi (not survived at 14 days pi).
2. Pathogenicity test analysis: For each group, the scores were pooled and the final score were the average of the pooled scores. All infected chicks in groups 2, 3, 4 and 5 representing groups infected with IBV isolates 4, 16, 18 and 23; respectively (table 5), signs of head and depression at 24 and 48 hours after virus inoculation were obtained. Sick chicks showed varying degrees of coughing, sneezing, tracheal rales, and watery feces (Fig 7, 8 and 9). The clinical scores were scored in (table 5). Obtained mortalities were 20%, 0.0 %, 0.0% and 100% with isolates 4, 16, 18 and 23; respectively (table 24). Pathogenicity index were 22, 18, 19 and 30 for isolates 4, 16, 18 and 23; respectively based on the necropsy of kidney and trachea of the survivor and dead chicks. So, isolates can be classified according to the pathogenicity index to highly, intermediat, highly and higly pathogenic for isolates code 4, 16, 18 and 23; respectively (table 6)(Fig 10 - 17). The main common lesions were swollen and pale kidneys together with tubules and ureters distincted by urate (Fig 35 and 36) (table 23). Recorded clinical scores were 2.25, 1.6 and 1.8 for isolates 4, 16 and 18; respectively. Score of isolate 23 was not

recorded, where all chicks were dead on day 5 and 6 pi (table 5).

3. Effect of IBV on body weight: As shown in table (7), IBV affected the performance of the infected chicks as judged by the body weight gain in groups infected with isolates 4, 16, and 18 where 49.9, 45.47 and 48.5 reductions in body weight percentage were obtained; respectively.
4. Microscopic kidney lesions; Principally, kidney lesions of IBV-infected chicks were of an interstitial nephritis. The virus caused granular degeneration, vaculation and desquamation of the tubular epithelium, and massive infiltration of hetrophils in the interstitium. The lesions in tubules was most prminent in the medulla. Inflammatory cell population, lymphocytes and plasma cells were seen (table 26) and Figs (37-41).
5. Microscopic tracheal lesions: The common findings in trachea of chicks infected at 1 day old with field isolates of IBVs were generally localized in the mucosa and lamina propria. The mucosa revealed variable degrees ranged from edema to mild or sever pronounced degeneration of the epithelial lining. Sometimes, goblet cells were activated and coalesce forming wide vacules.

The lamina propria revealed mild congested blood vesseles associated with hemorrhages, and infiltration with inflammatory cells. Concerning chicks infected with H120 live vaccine, pronounced activation of goblet cells was characterized.[table (8) and Figs (18-7. Results of reisolation: IBV could be isolated from organs collected from both dead and survived birds of groups 2, 3, 4 and 5 representing groups inoculated with IBV isolates 4, 16, 18 and 23; respectively.

**Table (4): Serological response of SPF chicks infected at 1 day old with IBVs and examined at 14 days age as Judged by ELISA (Synbiotic).**

IBV strain	Exam. No.	Descriptive Statistics						Post No.	Post %
		Min.	Max	Mean	GMT	SD	% CV		
4	8	0	1426	610	143	487	56.183	6	75
16	10	0	1506	697	340	490	45.23	9	90
18	10	0	1348	550	161	460	51.47	8	80
23	0	0	NT	NT	NT	NT	NT	NT	NT
H120	10	189	2020	746	568	589	48.543	10	100
Control	10	0	0	0	0	0	166.25	0	0

Exam = Examined

GMT=Geometric mean titer

No=Number

%=Percentage

Min=Minimum

CV=Coefficiance of variance

Max=Maximum

SD=Standard deviation

Post.=Positive

NT=Not tested (where 10 infected chicks dead on day 5, 6 pi)

\* Positive =Based on ELISA titer equal to or over 165 consider positive.

**Table (5): Clinical scoring of SPF chicks infected at 1 day old with IBVs and slaughtered at 14 day pi.**

Group	IBVs (isolates code)	Observation within 14 days post infection			Clinical score
		Infected No.	Survived No.	Dead No.	
1	Slaughtered (a)	10	0	0	NT
2	4	10	8	2	2.25
3	16	10	10	0	1.6
4	18	10	10	0	1.8
5	23	10	0	10(b)	NC
6	H120 Vacc	10	10	0	0
7	Control	10	10	0	0

(a) slaughtered at one day old for serological testing and proved negative by ELISA

(b) 5 chicks dead on day 5, and 5 chicks dead on day 6 post-infection.

Control = non infected      NT = not tested (slaughtered pre-experiment).

NC = not calculated, where chick were dead at 5 and 6 days pi.

Clinical score (*Avellaneda et al., 1994; Wang and Huang, 2000*) =

Score 0 = No clinical signs;

Score 1 = lacrimation, slight shaking of head, watery feces;

Score 2 = lacrimation, presence of nasal exudate, depression, watery feces;

Score 3 = strong (lacrimation, presence of nasal exudate, depression, severe watery feces).

**Table (6): Results of necropsy of SPF chicks infected at one day old with IBVs and examined survivor and dead during 14 days observation.**

IBV Isolate code	Necropsy record													
	Air Sacs					Trachea				Kidney			Heart	Liver
	No Lesion	Cloudiness	Moderate exudate	Thick exudate	Cheesy exudate	No Lesion	Slight increase mucin	Large increase mucin	Mucosal congestion	No lesions	Swelling	Urate	pericarditis	Perihepatitis
4	0	10	4	5	1	0	3	5	3	0	10	2	2	0
16	0	10	3	7	0	0	1	1	8	2	8	1	0	0
18	0	9	3	4	2	1	9	0	9	0	10	2	0	0
23	0	10	3	2	0	0	3	7	3	0	10	10	0	0
H120	8	2	0	0	0	8	2	0	0	10	0	0	0	0
Control	10	0	0	0	0	10	0	0	0	10	0	0	0	0

**Table (7): Pathogenicity index results based on necropsy of kidney and trachea of SPF chicks infected at 1 day old with IBVs and examined (survivors and dead) during 14 days observation.**

IBV Isolate code	No. infected	Observation record			Score		Pathogenicity index (a)	Pathotype(b)
		Dead	Survive	Mortality%	Kidney	Trachea		
4	10	2	8	20	10	10	22	High
16	10	0	10	0	8	10	18	Intermediate

18	10	0	10	0	10	9	19	High
23	10	10	0	100	10	10	30	High
H120-vacc.	10	0	10	0	0	2	2	Low
Control	10	0	10	0	0	0	0	-

Vacc.= Vaccine

Kidney and trachea score = No of chicks with lesion score of 1

(a) pathogenicity index = No of chicks with lesion score 1 + 1 point for every 10% mortality.

(b) pathotype = low (pathogenicity index value 1-9), intermediate (pathogenicity index value 10-18), High (pathogenicity index value 19)

**Table (8): Effect of IBVs on body weight of survivor SPF chicks infected at one day old and recorded at 14 day old.**

Item	IBV-isolated infected groups				
	4	16	18	H120	Control
Survivor No.	8	10	10	10	10
Range	90.66-130.44	99.57-133.71	86.81-135.53	200.2-218	202-218
Mean	105.71	115.57	108.74	210	210.9
BW%	50.1	54.56	51.5	99.5	100%
Reduction in BW%	49.9	45.47	48.5	0.5	0

$$BW\% = \frac{\text{Mean body weight of infected birds}}{\text{Mean body weight of non infected birds}}$$

x 100: Reduction in BW % = BW% of non infected group – BW% of infected group.

Chicks infected with IBV (isolate 23) not recorded (where all chicks dead on day 5 and 6 post infection).

Statistical analysis.

F-calculated = 31.801, significant at P < 0.001 using one way ANOVA test.

Duncan multiple range test for comparative of means (body weight)

Group		N	Subset for alpha = 0.05	
			1	2
1	IBV isolate 4	8	105.7138	
3	IBV isolate 18	10	108.7410	
2	IBV isolate 16	10	115.5730	
4	IBV H120	10		210.0000
5	Control	10		210.9000
Sig.			0.070	0.861

Means for groups in homogeneous subsets are displayed.

Data represent insignificant difference between H120 and control (subset 2) comparing with other tested groups.

**Table (9): Results of Histopathological lesions in kidneys associated with infection of one day old SPF chicks with IBVs and examined (survivors) at 14 days pi.**

IBVs Isolate code	Oedema	Degeneration	Necrosis	Inflammatory cells	Urates	RER
Isolate No. 14	++	+++	++	++	-	++
Isolate No. 16	+	+	+	++	-	+
Isolate No. 18	+	++	+	+	+	+
H120 (Vacc.)	+	+	-	+	-	+

Vacc.= Vaccine

Oedema = swelling of infected epithelial cells.

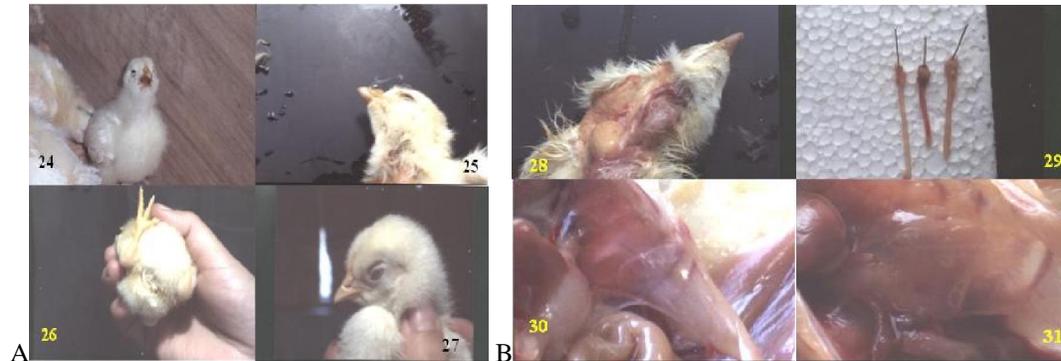
Degeneration = granular degeneration of tubular epithelium.

Necrosis = focal area of necrosis in tubular epithelium.

Inflammatory cells = tubular epithelium, mostly in medulla infiltrated with inflammatory cells (Hetrophils, Lymphocytes and plasma cells).

Urates = ureters distended with urates and sometimes with casts.

RER = increase the amount of rough endoplasmic reticlm (RER).



#### A. Clinical signs developed in experimental pathogenicity testing.

**Fig. (24):** Severe gasping of SPF chick developed within 2 days after infection with field isolate of IBV.

**Fig. (25):** Frothy nasal exudates and nasal discharge.

**Fig. (26):** Watery feces as Judged by soiled vent feather. **Fig. (27):** Wet eye.

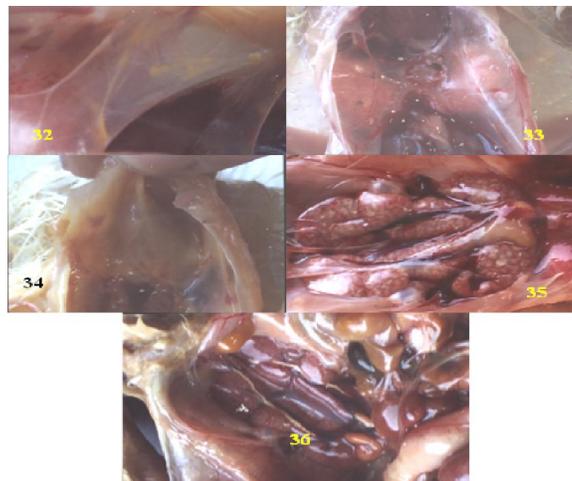
#### B. Gross lesions developed in experimental pathogenicity testing.

**Fig. (28):** Congested trachea after infection developed in experimental chicks.

**Fig. (29):** Trachea revealed different degrees of congestion.

**Fig. (30):** Thoracic air sac showing turbidity and cloudness.

**Fig. (31):** Frothy thoracic air sac developed in dead SPF chick after infection with field isolate of IBV.



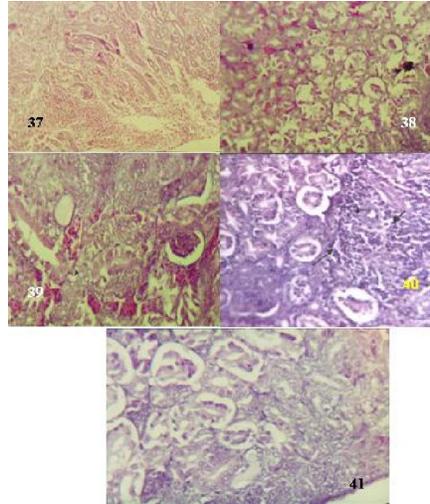
**Fig. (32):** Cloudy and yellowish thoracic air sac developed in dead SPF chick after infection with field isolate of IBV.

**Fig. (33):** Lung with focal area of pneumonia and turbid thoracic air sac.

**Fig. (34):** Pericarditis and yellowish exudates in thoracic air sac.

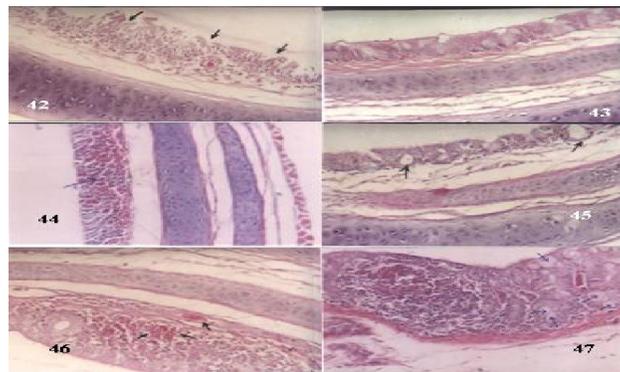
**Fig. (35):** Pale kidney with nephritis and deposition of uric acid in ureters of survived SPF chick after 14 days of infection with field isolate of IBV.

**Fig. (36):** Deposition of uric acid in ureters of survived SPF chick after 14 days of infection with field isolate of IBV.



**Histopathological findings of kidney of infected 1 day old SPF chicks with IBV (No.16 & 18) and examined (survivor) at 14 days pi.**

- Fig. (37):** Severe congestion and hemorrhages within tubular epithelium, mostly in medulla in chicks infected with IBV (No.16) (H & E. x 100).
- Fig. (38):** Severe degenerative changes of renal tubules in chicks infected with IBV (No.16) (H & E. x 100).
- Fig. (39):** Marked degenerative and undifferentiated renal tubules in chicks infected with IBV (No.18) (H & E. x 100).
- Fig. (40):** Aggregation of inflammatory cells (heterophils, lymphocytes and plasma cells) in tubular epithelium, mostly in medulla in chicks infected with IBV (No.18) (H & E. x 250).



**Histopathological findings of trachea of infected 1 day old SPF chicks with IBV (18,16 and H 120) and examined (survivor) at 14 days pi.**

- Fig. (42):** Mucosa of trachea infected with IBV (18) showed degenerative changes in epithelial lining cells (H & E. x 250).
- Fig. (43):** Mucosa of trachea infected with IBV (No.16) revealed degeneration of epithelial lining and activation of goblet cells (H & E. x 250).
- Fig. (44):** Lamina propria of trachea infected with IBV (No.16) have severe hemorrhages (H & E. x 250).
- Fig. (45):** Activated goblet cells of trachea infected with IBV (No.16) (H & E. x 250).
- Fig. (46):** Hemorrhages in lamina propria of trachea infected with IBV (No.18) (H & E. x 250).
- Fig. (47):** Activated goblet cells of trachea vaccinated with H120 (H & E. x 250).

The possibility of the protection provided by live attenuated IB vaccine against challenge with field

IBV isolates. To evaluate the protection of the

respiratory tract provided by live attenuated IBV vaccine against challenge with field IBV isolates.

ELISA then the remaining birds were divided as follow:

Seventy, one day old SPF chicks were used, 10 chicks were sacrificed for serological examination by

**Table(10).Experimental design ( for cross protection study).**

Group	Subgroup	no. of birds	Treated groups/ isolate No		
			1day old	4weeks old	
Group A	Subgroup A1	6	H120 Vac.	Isolate 4	Vaccinated Challenge Groups
	Subgroup A2	6	H120 Vac.	Isolate 16	
	Subgroup A3	6	H120 Vac.	Isolate 18	
	Subgroup A4	6	H120 Vac.	Isolate 23	
	Subgroup A5	6	H120 Vac.	PBS	Vaccinated non challenge control
Group B	Subgroup B1	6		Isolate 4	Non Vaccinated Challenge Groups
	Subgroup B2	6		Isolate 16	
	Subgroup B3	6		Isolate 18	
	Subgroup B4	6		Isolate 23	
	Subgroup B5	6		Sterile PBS	Non vaccinated non challenge control

Results in which various heterologous IBV strains were used for challenge of 4 weeks old SPF chickens vaccinated at one day old by H120 vaccine are summarized in table (11): H120 vaccine protected poorly against challenge with IBV isolates 4, 16, 18 and 23 where protection percentage were 8.1, 55,

Clinical signs percentages (in vaccinated challenged groups) were 100, 50, 100 and 100, for IBV isolates 4, 16, 18 and 23; respectively (table, 30). 20% mortality was observed in non vaccinated group challenged with isolate code 23 (table 10).

10.5 and 12.6; respectively (table 11). In this the higher the score, the better the level of protection achieved.

Complete ciliary activity of trachea either by EM scanning or inverted microscope examination is presented (Fig. 48 and 51) and complete ciliostasis (Figs, 49 and 52) and partial ciliostasis (Fig.,50). Serology results are shown in (table )

**Table (11): IB antibody titre of chicks at 1 day old (pre-experiment) and 28 days (pre-challenge from groups A and B) as judged by ELISA (synbiotic).**

Group	No. of samples	Age/day	Treatment	Descriptive statistics						Post No.	Post %
				Min	Max	Mean	GMT	SD	%CV		
Pre-experiment	10	1	No treatment	0	0	0	0	0	81.04	0	0
Group A	10	28	Vacc H120 at 1 day old	1412	3804	2395	2249	878	22.73	10	100
Group B	10	28	Non vaccinated at 1 day old	0	0	0	0	0	34.85	0	0

No = Number    % = Percentage    Min = Minimum    CV = Coeffiencie of variance    Max=Maximu

**Table (12): Development of clinical signs and mortalities during 5 days postchallenge with IBVs at 4 weeks of age in SPF chicks (vaccinated and non vaccinated at 1 day old with live H<sub>120</sub> vaccine).**

Item	Group									
	A Vacc/chall				B Non vacc/chall				C = control groups	
Subgroup	A1	A2	A3	A4	B1	B2	B3	B4	A5	B5

Number	6	6	6	6	6	6	6	6	6	6
IBV-challenge strain	4	16	18	23	4	16	18	23		
Signs: d1-pc	0	0	1	0	0	0	0	1	0	0
d2-pc	0	0	2	3	6	2	2	5	0	0
d3-pc	2	1	6	6	6	6	6	6	0	0
d4-pc	6	3	6	6	6	6	6	5(a)	0	0
d5-pc	6	3	6	6	6	6	6	4(b)	0	0
Deaths-No.	0	0	0	0	0	0	0	2	0	0
Sign %	100	50	100	100	100	100	100	100	0	0
Mortality%	0	0	0	0	0	0	0	33.3	0	0

d = day, pc = post-challenge, vacc = vaccinate, chall = challenge,

A5 = vaccinated non challenge. B5 = Non vaccinated non challenge

clinical signs = include one or more signs of depression, lacrimation, slight shake head, swollen head, soft dropping, respiratory signs.

(a) one chick was died in d4PC (remaining 5 chicks)

(b) One chick was died in d5PC (remaining 4 chicks)

**Table (13): Results of cross protection test as Judged by tracheal ciliary activity.**

Item	Group A (vaccinate/challenge)				Group B (non vaccinate/challenge)			
	Sub-group A				Sub-group B			
	A1	A2	A3	A4	B1	B2	B3	B4
Vacc.	H120	H120	H120	H120	-	-	-	-
IBV-Chall	4	16	18	23	4	16	18	23
No. Exam.	6	6	6	6	6	6	6	4(a)
(b) Mean ciliostasis score	36.3	16.65	33.8	34.3	39.5	37.0	37.8	39.3
Protection%	8.1	55	10.5	12.6				

IBV. Chall = IBV challenge strain. Vacc = Vaccination

Group A = vaccinated at 1 day old with IBV-vaccine (H<sub>120</sub>), challenged at 28 day old.

Group B = Non vaccinated, challenged at 28 day old.

IBV-challenge strain: isolate 4 (A<sub>1</sub>, B<sub>1</sub>), isolate 16 (A<sub>2</sub>, B<sub>2</sub>), isolated 18 (A<sub>3</sub>, B<sub>3</sub>), isolate 23 (A<sub>4</sub>, B<sub>4</sub>)

(a) Two chick found dead (not examined)

protection score % =  $\left[ 1 - \frac{\text{mean ciliostasis score for vacc-chall-}}{\text{group}} \right] \times 100$

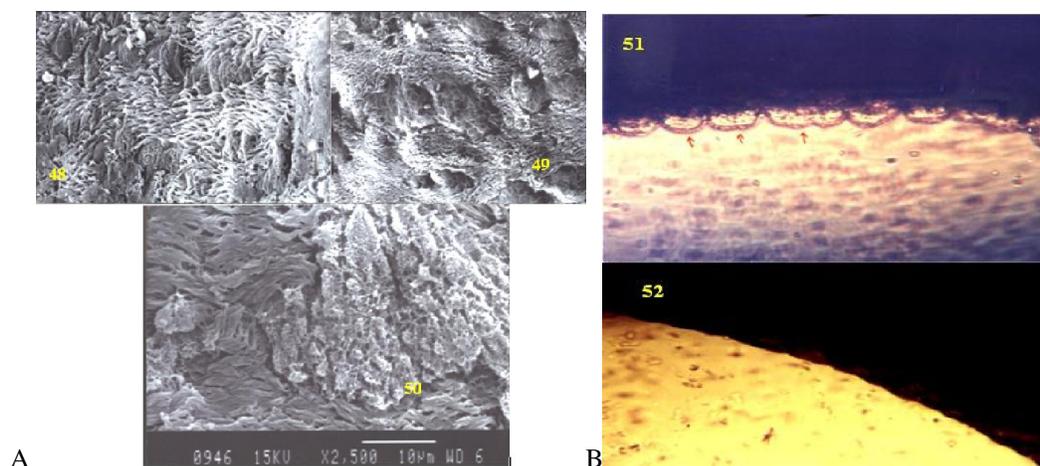
#### Statistical analysis

	Isolate 16	Isolate 4	Isolate 18	Isolate 23
Subgroup 1	55			
Subgroup 2		8.1	10.5	12.6
Fischer exact value	23.124*			

\* Significant at P < 0.05 using Fischer Exact Probability test for comparative of means.

Data significant divided into two significant subgroups where subgroup 1 (isolate 16), significant different than subgroup 2 (isolates 4, 18 and 23)

using Duncan Multiples range test for comparative of means.



#### A. Electron microscopy scanning (EMS) for trachea

**Fig. (48):** Trachea of control chicks. Complete ciliary activity.

**Fig. (49):** Trachea of non vaccinated-challenged chicks. Complete ciliostasis.

**Fig. (50):** Trachea of vaccinated-challenged chicks. Complete ciliary activity (left) and complete ciliostasis (right).

#### B. Breadth of protection of respiratory tract provided by live attenuated IB vaccine (H 120) against challenge with IBV of heterologous serotype.

**Fig. (51):** Complete presence of tracheal cilia in non-infected SPF chicks as demonstrated with inverted microscope.

**Fig. (52):** Ciliostasis with complete detachment of tracheal cilia of SPF chick, 4-days post-challenge with IBV (isolate 16) as demonstrated with inverted microscope.

#### 4. Discussion

The IBV genome consists of approximately 27 kb (Bournell et al., 1987) and codes of three structural proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein, and the nucleocapsid (N) protein (Lai and Cavanagh, 1997; Enjuanes et al., 2000). In addition, a fourth protein (small membrane protein, E) is believed to be associated with the virion envelope in very small amounts; it is essential for virus particle formation (Cavanagh and Naqi, 2003). The virus has a world wide distribution, and many variants have been isolated (Davelaar et al., 1984; Wang et al., 1994; Liu et al., 2003). The appearance of antigenic variants of IBV cause a major problem in the poultry industry. Natural outbreaks of IBV are controlled through the use of vaccines. In spite of the routine use of vaccines, IBV outbreaks continue in vaccinated fowls (Gelb, 1989; Wang and Tsai, 1996; Liu et al., 2003). The S protein comprises two or three copies of each of two glycopeptides, S1 and S2 (approximately 520 and 625 amino acids; respectively). Hemagglutination-inhibiting (HI) and most of the neutralizing (VN) antibodies are induced by S1 (Cavanagh et al., 1988; Koch et al., 1990; Jackwood et al., 1992; Ignjatovic et al., 1997).

As a result of molecular studies, it was now known that it was the S1 part of the IBV that is

responsible for determining its serotype (Cook et al., 1999). Furthermore, a new IBV serotype could arise as a result of only a very few changes in the amino acid composition of the S1 part of the spike protein (Cavanagh et al., 1992b), with the majority of the virus genome remaining unchanged. The greatest divergence in the amino acid sequence was concentrated between the residues 53 and 148 of the S1 (Niesters et al., 1986; Wang et al., 1994). Two hypervariable regions (HVR) within the S1 at positions 56-69 and 117-133 from the beginning of the S1 were also identified (Niesters et al., 1986). The HVR was an essential determinant of coronavirus serotype specificity (Cavanagh and Naqi, 2003).

Strain classification or, more appropriately, serotype or genotype classification of IBV is based on feature of the S protein. The many ways that are used to differentiate isolates of IBV have been thoroughly compared by De Wit (2000). Traditionally, IBV serotypes have been defined by VN tests, VN antibody being induced by the S1 subunit of the S protein. VN tests are time-consuming, especially because an increasing number of standard sera, corresponding to different serotypes, are included in analysis, and in some cases uncertain classification of serotypes of field isolates results from one-way neutralization did not induce a clear

cut classification (Song et al., 1998). Non serological differentiation methods such as polyacrylamide gel electrophoresis (Cowen and Hitchner, 1975), nucleic acid hybridization (Cavanagh, 1989), and oligonucleotide fingerprinting (Kusters et al., 1987) were not hampered by the appearance of new IBV isolates and resulted in an objective typing system. These methods were, however, complex and labor-intensive and required large amounts of highly purified virus particles.

RT-PCR has been described previously using IBV RNAs extracted from allantoic fluid, and this technique has been shown to be very efficient for the detection of IBV and for the identification of IBV types (Adzhar et al., 1996; Jackwood et al., 1997; Keeler et al., 1998; Handberg, et al., 1999; Meulemans et al., 2001). Laboratories are using Reverse transcriptase polymerase chain reaction (RT-PCR), usually the S1 part of the S protein gene, followed by restriction endonuclease analysis or sequencing. Such nucleic acid approaches then define IBV isolates by genotype rather than by serotype.

In our study; in order to use a general method for the detection of IBV strains by a common RT-PCR, we used oligonucleotide pairs based on 3' untranslated (UTR) sequence. The 3' untranslated (UTR) sequence, unlike the remainder of the IBV genome apart from the 5' leader, is present on all IBV RNAs. This sequence is the most abundant IBV sequence in RNA extracted from infected allantoic fluid. Therefore, one would expect to obtain maximum sensitivity in the RT-PCR when using oligonucleotide pairs based on 3' (UTR) sequence (Adzhar et al., 1996). We extended the sensitivity of the PCR, by RNA purification prior to the RT-PCR reaction to remove nonspecific inhibitors. The oligonucleotide pair was applied to RNA extracted from allantoic fluid harvested from SPF embryos at the level of fifth embryonic passage inoculated with 25 IBV field isolates (found positive in AGP test). By use of this oligonucleotide pair, IBV was detected in all these samples where 298 bp product was detected, each containing one of the 25 IBV strains. This result accords with Adzhar et al., (1996); Jackwood et al., (1997); Keeler et al (1998); Handberg et al., (1999); Meulemans et al., (2001), and confirm the previous detection of IBV by the AGP-test performed on CAM of inoculated eggs. This result indicated that the oligonucleotide pair is universally applicable on IBV strains and therefore provided a useful tool for detection and identification of IBV isolates (Handberg et al., 1999). This findings confirm the prevalence of IBV in chicken farms since the initial report in Egypt (Ahmed, 1954), followed by several publications concerning the isolation of IBV (Eissa et

al., 1963; Ahmed, 1964; Amin and Moustageer, 1977; Sheble et al., 1986; Bastami et al., 1987; Mousa et al., 1988; El-Kady, 1989; Mahmoud, 1993; Ahmed, 2002; Abdel Moneim et al., 2002; Madbouly et al., 2002; Sultan et al., 2004; Lebdah et al., 2004; Sediek, 2005).

Antibody based tests for identifying IBV isolates included virus neutralisation (VN) (Cowen and Hitchner, 1975), hemagglutination inhibition (HI) (Alexander and Chettle, 1977), and the use of S1 specific monoclonal antibodies (Karaca et al.,1992). More recently, tests directed at reverse transcription - polymerase chain reaction (RT-PCR) amplification of S1 have become more commonly used because their short turn-around time and high degree of specificity (Kingham et al., 2000).

Direct automated cycle sequencing (DACS) strategies had broad applications in research and diagnostics. The development of DACS procedures were used to diagnosis and study the epidemiology and evolution of viral diseases significant, particular in case of viruses that exhibit antigenic variability (Kingham et al., 2000). The application of S1 – sequence analysis for epizootiological studies of IBV has been proposed. DACS provided sequence information in several days and was applicable to large number of IBV isolates (Kingham et al., 2000).

The most frequently published IBV sequences in Gene Bank are localized at the S1 gene, which is a part of the IBV genome with high variability. Therefore, it provided obvious possibilities for the construction of strain-specific oligonucleotides. Our investigation was designed to identify incidence of three serotypes of IBV (determined according to recent published data in Egypt), we used oligonucleotide primer designed by Adzhar et al.,(1997) which was capable to detect and differentiate three serotypes of IBV (Massachusetts, D274 and 4/91). Massachusettes strain was selected to detect vaccine virus and to cease investigation of a field samples if such virus was shown to be present, D274 was selected as it was reported as dominant variant strain in Egypt in 1980-1991 (Bastami et al., 1987, El Kady, 1989, Madbouly et al., 2002), and 4/91 was selected based on the report of Sultan, (2004) who isolated 6 IBV isolates from white commercial egg laying chickens flocks aged 12-28w, one of them was identified as 4/91 related serotype. When applied this specific primer to infectious allantoic fluid, the oligonucleotide pairs identified the IBV variant strains for four IBV isolates for which they were designed where 385 bp product was detected and none of the others (although, other types might have been present but not detected). Therefore it was believed that these oligonucleotide were type

specific and may be used for epidemiological surveillance rather than for primary diagnosis of IBV (Handberg et al., 1999).

Results of S1 sequence analysis of isolate code (4) showed high nucleotide similarity to isolate CK/CH/LDL/011 isolated in China (100% nucleotide identities), and Q1 isolate isolated in Singapore (100% nucleotide identities).

S1 sequence analysis of isolate code (16) revealed its close relatedness to Massachusetts serotype. It showed high nucleotide similarities to M41 (97% nucleotide identities), Connecticut (98% nucleotide identities), Beaudette (97% nucleotide identities), Egypt/F/03 (96% nucleotide identities), and GX1-98 (96% nucleotide identities). This finding agreed with that reported by Abdel-Moneim et al., (2006).

S1 sequence analysis of isolate code (18) showed the isolate was matched with isolate 720/99 isolated in Israel (98% nucleotide identities), and with isolate 885 isolated in Israel (98% nucleotide identities). This finding in agreement with that reported by Abdel-Moneim et al., (2002).

S1 sequence analysis of isolate code (23) showed that isolate was matched with isolate FR/94049-94 isolated in France (98% nucleotide identities), isolate Spain/92/S1 isolated in Spain (96% nucleotide identities), and with strain 4/91 (93% nucleotide identities). This finding accord with the finding reported by Sultan, (2004).

The majority of the variant were isolated from north of country (isolate code 4, 16, 18 and 23 were isolated from Kalubia, Dakahlia, Dakahlia and Giza; respectively). Therefore, it is not possible to draw any conclusion regarding the geographic distribution of the different antigenic type through the country. It is clear, however, that 3 different IBV types are coexisting in the north part of Egypt, an area that has a high poultry density.

Results indicated that one isolate code (16) was genetically related to the Mass type of IBV, while the other three isolates were not genetically related to the Mass type of IBV and seemed to be newly introduced pathogens in poultry population in Egypt. Variant strains of IBV were detected in vaccinated broiler flocks four or more weeks old, this can be explained by Massachusetts vaccine strains would have been replicating in high proportion of the birds during the first week. This may have competed variant field virus during the first few days after vaccination. This delay may be expected while the respiratory mucosae recovered from the replication of the Massachusetts vaccine. Possibly, some immune responses, including non-

specific ones, to the Massachusetts type vaccine would impede replication of variant type (Cavanagh et al., 1999).

The relationship between serotype and pathogenicity of IBV was not well understood. But as genetic variation result in serotype changes, it may result in changes in tissue tropism and pathogenicity of the virus that lead to generation of new IBV serotypes (Yu et al., 2001). Other than knowing the serotype for classification purposes, it was important to know pathogenicity of the challenge strain in order to choose the vaccine with the antigenicity and immunogenicity needed (Avellaneda et al., 1994). The pathogenicity of different strains of IBV had been difficult to assay. Signs of respiratory infection were usually the only indication seen in birds with the disease, offering only a subjective and imprecise measure of virulence. When a secondary bacterial infection occurs, a common characteristic of IBV infection is the development of airsacculitis, pericarditis and perihepatitis. These features has been reproduced experimentally (Fabricant and Levine, 1962).

In the present study four IBV isolates which were characterized as variants, were examined further to evaluate their pathogenicity, day old SPF chicks were selected because we expected them to be most susceptible to infection at this age and they were also free of antibodies titer against IBV as well as free from other infectious agents (Dhinkar and Jones, 1996a). Three IBV isolates (4, 16 and 18) were capable to induce respiratory signs with clinical score of 2.25, 1.6 and 1.8; respectively. Also, respiratory lesions (air sacs and trachea) and renal (kidney) lesions were obtained. These findings agreed with Ignjatovic and Sapats (2000), who reported that strains of IBV differ in virulence or pathogenicity for the respiratory tract, kidney or oviduct. Although the virulence of many IBV strains had not been clearly defined, examples illustrated the predominant feature of each pathotype. The majority of IBV strains, including those of the Massachusetts (Mass) serotype, of which the M41 is the representative strain, produce prominent respiratory disease as recorded by Cavanagh and Naqi, (1997). Most of these strains do not induce mortality when acting alone. However, in experimental infections, variable mortality rates are obtained, indicating the differing pathogenic potential of strains to predispose chicks to the development of airsacculitis, pericarditis and perihepatitis as similar to Smith et al., (1985).

Concerning the capability of IBV field isolates to induce mortality, only two IBV isolates (4 and 23) were able to produce 20% and 100% death; respectively. The high mortality rate observed in one

day old chick that experimentally inoculated with isolate code 23 compared to mortality pattern in the original flock (layer-41 week old) can be explained by the fact that the most severe clinical response of IBV appear in very young chicks and as age increases chickens become more resistant to IBV induced mortality (Smith et al., 1985). These findings accord with Wang et al., (1996), who reported that IBV alone in experimental infection could cause death after infection ranged from 10, 20, 50 and 60 percent in experimental infected chicken groups.

It was well documented that IB had a significant economic impact in broilers whereas production losses may due to poor weight gains (Ignjatovic and Sapats, 2000). Our results found, where severe losses in broiler weights on a comparison of control group as a sequence of infection with IBV field variant isolates coded 4, 16 and 18 reached 49.9%, 45% and 48.5%; respectively. This may be explained as the affected chicks suffered from depression and marked reduction in the feed consumption which result in significant loss of body weight from 3 days after infection (Otsuki et al., 1990).

Some IBV strains either caused nephrosis-nephritis in young birds or else contributed to urolithiasis in layers (Cowen et al., 1987). Three of our IBV isolates were nephropathogenic strains, where they induced gross renal urate deposition and histological lesions in the experimental chicks. Similarly, variants of IB were reported as nephropathic, M41, 720/99 Israil, D274, D3896, D311, D3896, D1559, D3128, 4/91 (Bastami et al., 1987; Eid, 1998; Abdel-Moneim et al., 2002; Madbouly et al., 2002; Lebdah et al., 2004; Sultan et al., 2004; Sediek 2005). Nephropathogenic strains have been a predominant IBV pathotype only in Australia (Cumming, 1969b), with sporadic isolation in other countries. However, during the last decade, nephropathogenic strains have emerged in many countries including Italy, the USA, Belgium, France, China and Japan (Butcher et al., 1989; Wang et al., 1996). In some countries of Europe, these strains have become the predominant pathotype of concern (Meulemous et al., 1987). The nephropathogenic IBV strains were able to induce mortality, principally in chicks under the age of ten weeks and differ markedly in virulence, with variable mortalities of between 5% and 80% in experimental infections. This may explain mortality observed in experiment after inoculation of the variant IBV isolates code (4 and 23) as deaths may be resulted from acute renal failure. Changes accompanied with kidney infection with IBV were documented. The serum ions content were affected by the change in electrolyte balance in

the kidney and the intake of ions in feeds. The major change in electrolyte balance in the kidney is the increased output of sodium in the urine, which is associated with diuresis (Condron and Marshall, 1985). The ability to reabsorb sodium in infected birds might have damaged, which subsequently led to the low sodium content in the blood. Both the intracellular and renal luminal potassium concentration in IBV-infected birds were lower than those of normal birds (Condron and Marshall, 1991). The higher potassium content in the blood in infected birds might possibly be a consequence of the leakage of potassium from damaged cells and secretory damage. This explain the severe watery feces observed in groups inoculated with variant IBV isolates as this watery feces result from polyurea.

In regard of histological changes of the kidney, IBV is the only member of coronaviridae family reported to possess nephropathogenic effects whereas renal epithelial cells containing IBV particles were numerous in the tubular epithelium. The infected epithelial cells in the lower nephrons and ducts showed a marked increase in the amount of rough endoplasmic reticulum (RER), indicating higher susceptibility to the present virus infection. These findings support the view that the production and maturation of coronaviruses are dependent on the structure and function of RER (Klumperman et al., 1994). Virus formation by budding in IBV-infected renal epithelial cells is mostly from membranes of RER (Chen and Itakura, 1996). Physiological studies were recorded in which elevation of plasma uric acid, increasing the urinary water losses beside lowering of urine osmolality in the IBV-infected chickens. The dilated endoplasmic reticulum in the renal epithelial cells may represent changes in water and ion transport. The present structural changes in IBV-infected epithelial cells, primarily in the lower nephrons, might indicate that fluid and electrolyte transport were impaired, and thus responsible for the renal failure then death (Chen and Itakura, 1996).

Concerning histopathological lesions in trachea associated with IBV infection in 1 day old SPF chicks and examined at 14 days pi, the findings in the mucosa revealed edema, sloughing and degeneration of epithelial cells and activation of goblet cells, which agreed with the findings of Cavanagh and Naqi (2003). The lamina propria was characterized by massive infiltration by lymphoid inflammatory cells, a result that have been already obtained and reported by Cavanagh and Naqi (2003), as features which can take place in trachea of birds infected with IBV.

Serum samples taken from SPF chicks 14 days post inoculation with 3 variant IBV isolates code (4,

16, and 18) and subjected to ELISA test (isolate code 23 could not be examined by ELISA as all chicks died before 14 days of age) showed geometric mean antibody titer lower than 500 which is extremely low for all groups, this may be explained by the short time between infection and detection of antibody response (Avellaneda et al., 1994). But generally the occurrence of low seroconversion in all groups after the inoculation of variant IBV isolates is similar to the finding reported by Gough et al., (1992) who reported that variant IBV strains showed slow seroconversion.

IB was controlled primarily by using attenuated live virus vaccine (e.g., H<sub>120</sub>, Ma5, commercially available and registered in Egypt) as well as inactivated oil emulsion vaccine, but more than sixty serotypes of IBV have been reported from all over the world (Ignjatovic and Sapats, 2000). So, it is useful for implementation of control measure to determine which IBV serotype(s) have been circulating in region as, protection provided by vaccination with a vaccine of a given serotype, is directed mainly against homologous serotype and less against strains of other serotypes (Davelaar et al., 1984).

In the present study IBV isolates which were characterized as variants, were isolated from flocks had been vaccinated against the IBV using the H120 vaccine which makes the efficacy of such vaccination questionable. By performing *in vivo* protection study, it was possible to demonstrate the breadth of protection that currently available live IBV H120 vaccine (registered and applied in Egyptian market) can provide against challenge with a variety of IBV isolates of several other different genotypes (code 4, 16, 18 and 23) raised in the present work by molecular studies. The vaccine was administered by oculo-nasal route in order to ensure that each chick received the required dose of vaccine (Cook et al., 1999). Generally, three main approaches to the assessment of protection have been (1) observation of clinical signs; and removal of trachea at 4 or 5 days after challenge followed by either (2) quantitative assessment of ciliary activity or (3) detection of live challenge virus, usually by inoculation of embryonated eggs (Cavanagh, 2003). The second and the third methods result in similar deductions being made as regards protection (Marquardt et al., 1982). In our study we used observation of clinical signs followed by quantitative assessment of ciliary activity at 5 days after challenge for evaluation of protection as reported by Cook et al., (1999). Clinical signs percentages observed on chicks in groups challenged with isolates code 4, 16, 18 and 23 were 100%, 50%, 100%, 100% ; respectively. Also, protection

percentages based on the quantitative ciliary activity were 8.1%, 55%, 10.5% and 12.6%. Mortality percentage 20% was observed only in the group challenged by isolate code 23. This result indicated that H120 could provide only partial protection against challenge with isolate code 16 only, while provide only little protection against the challenge with other 3 isolates. This can be explained by isolate code 16 was related to M41 strain by the S1 sequence (97% match with M41 strain), so H120 (Mass serotype) could provide partial protection against it. Regarding the remaining 3 isolates codes 4, 18 and 23 based on the S1 sequence, there were no homology reported between them and the vaccine used so the vaccine provide only little protection against them. This results in agreement with finding that the reports of *in vivo* cross protection often declines with decreasing S1 sequence homology between vaccinal strain and strains used in challenge (Gelb et al., 2005).

Conclusively, the vaccination programme used in these trials resulted in poor protection of the respiratory tract against challenge with the four new IB serotypes that have been isolated recently in the present work from outbreaks in various governorates in Egypt.

The concept of protectotypes has been suggested to be a valuable one to consider in terms of developing strategies to control IBV infections (Lohr, 1988). The results presented here confirm its value and indicate it to be more relevant in this context than knowing the serotype of a new IB isolate. Rather than spending time determining its serotype, it is probably of more practical relevance in term of control strategies to perform protection studies with the isolate and determine the optimum vaccination programme to protect against it (Cook et al., 1999).

From the above mentioned results, it is clear that H120 live vaccine only is poorly protected against at least four existing new IB serotypes in Egyptian chicken farms, and as a result economic losses will be continuous. Using of new serotype(s) of vaccine to face the present status is advisable, in addition to shift of vaccination programmes is highly recommended either as importation of suitable existing serotype(s) or locally prepared from the present existing serotype(s). In spite, IB still threat for poultry production, where re-emerged variant(s) still occur.

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