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The prevalence of Infectious Bronchitis (IB) outbreaks in some chicken farms: II. Molecular characterization of field isolates of IB virus

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Twenty five isolates of IBV were isolated from 36 broiler and layer chicken farms collected from 13 governorates during 2 years started from January 2003. All the examined farms were vaccinated using the commercial live IB-H_{120} vaccine in addition to the IB-inactivated vaccine in the layer farm. The viruses were isolated and identified previously by chicken embryo, CEK cell culture inoculation. Isolates subjected to RT-PCR. Four isolates; three broiler farms and one from layer farm were genotyped using S1 partial gene sequencing. Typing of the four isolates using S1 partial gene sequencing, revealed that the isolated IBV strains showed homology to Asia, Europe, USA and Middle East strains.

IBV, is a prototype of family *Coronaviridae*, It contains 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 subunites (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).

Serotype 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 serotype 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).

Materials and methods

Viruses of IB.

Field isolates. They were obtained previously from 36 chicken farms showing symptoms suspected to be IBV infection.

Oligonucleotide primers. Primers for UTR and S1 were designed according to (Adzhar *et al.,* 1996 ; Cavanagh *et al.,* 1999) (Table 1). S1 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.

RNA extraction. RNA was extracted from the allantoic fluid from previously isolated field isolates using Qiagen viral RNA extraction kit according to the manufacturer instructions.

Reverse transcriptase polymerase chain reaction (RT PCR). RT PCR was performed on the RNA of the examined isolates . RT PCR amplicon was detected in 1.0% agarose *(Hispangar)*, according to (Adzhar *et al.,* 1996). DNA of amplicons were gel purified and used for direct sequencing.

Table (1): Primers for amplification of UTR and S1 by RT-PCR assay.

Primer	Sequence (5`-3`)	Gene	Location	*Tm*	Location reference
UTR2+	AAGGAAGATAGGCATGTAGCTT	3` UTR	234-255	56°C	Williams *et al.,* (1993)
UTR1-	GCTCTAACTCTATACTAGCCTAT	3` UTR	509-531		Williams *et al.,* (1993)
XCE1+	CACTGGTAATTTTTCAGATGG	S1	728 to 749	50°C	Adzhar *et al.,* (1997)
XCE3-	CAGATTGCTTACAACCACC	S1	1093 to 1111		Adzhar *et al.,* (1997)

Detection IBV and sequencing of a piece of the S1 Gene. It was done according to (Gelb *et al.,* 2005) using BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystem catalog No. 4337450).

Results

RT PCR for detection of IBV 3 UTR gene. Results revealed that 25 IBV-field isolates was positive for UTR motif using RT PCR.

Detection of IBV and partial S1 sequencing. Results revealed that the S1 gene was yielded at 385 bp (Fig.2 and Table 2).

Sequence analysis. Broiler isolate code (4) was found to be identical (100% homology) to Chinese isolate; CK/CH/LDL/011 and Singapore isolate Q1. Broiler isolate code (16) showed 98% homology to Connectcut strain, 97% to M41 strain., 96% with Egypt/F/03 and 96% with the Chinese isolate GX1-98. Broiler isolate code (18)showed 98% homology to isolates; 720/99 and 885 isolated from Israel. Layer isolate coded (23)showed 98% homology to the French isolate FR-94047-94 and 96% to isolate Spain/92/51 (Table 3 and Fig.2-6).

Discussion

The IBV genome consists of approximately 27 kb (Boursnell *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 worldwide 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.,* 1992), 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 is an essential determinant of coronavirus serotype specificity (Cavanagh and Naqi, 2003). Strain classification or, more appropriatey, 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 iduced 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

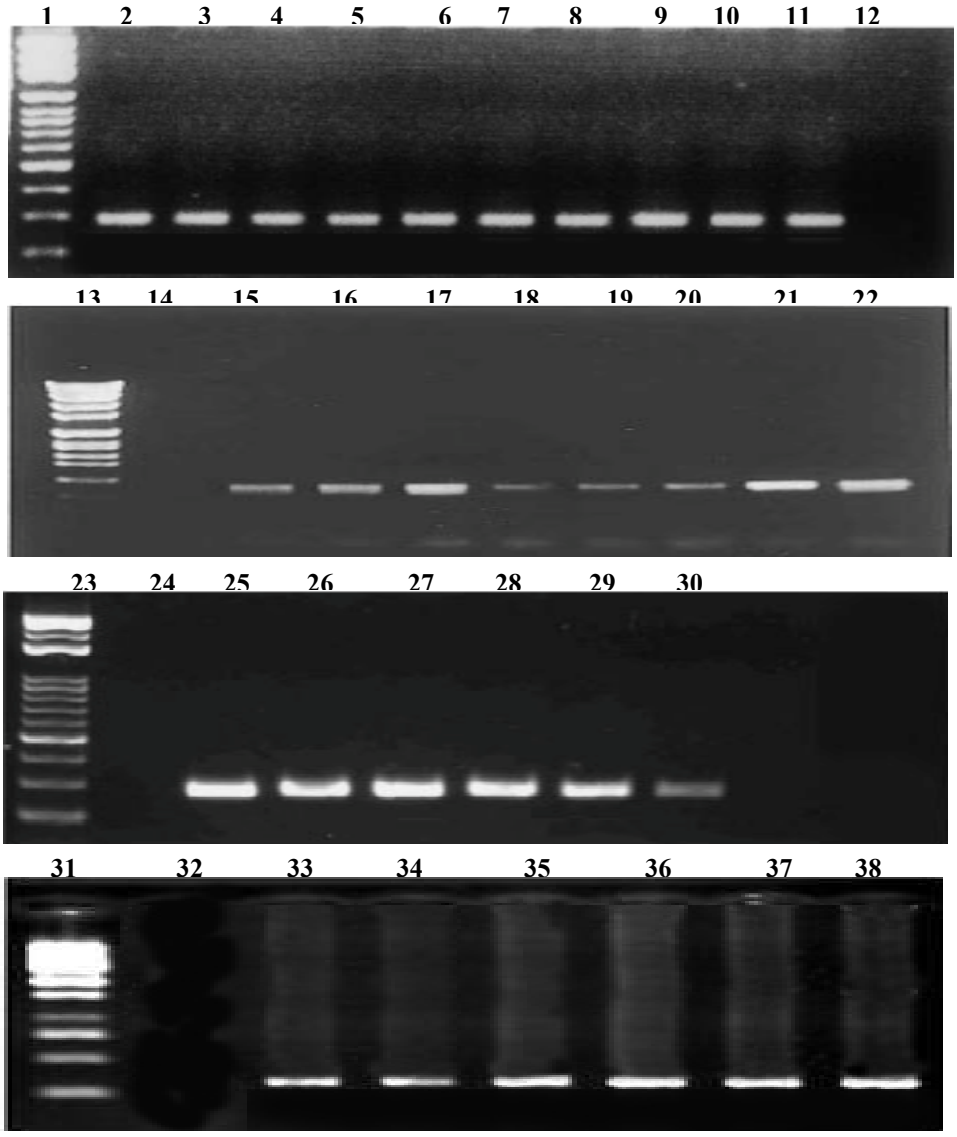

Fig. (1): RT-PCR for 25 IBV-tested AAF of egg embryos. Lanes 1, 13, 23, 31 marker. Lanes 12, 14, 24, 32 Negative Control. Lanes 11, 22, 30, 37, 38 positive control. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, 20, 21, 25, 26, 27, 28, 29, 33, 34, 35 and 36 field IBV tested samples with 298 bp-PCR-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

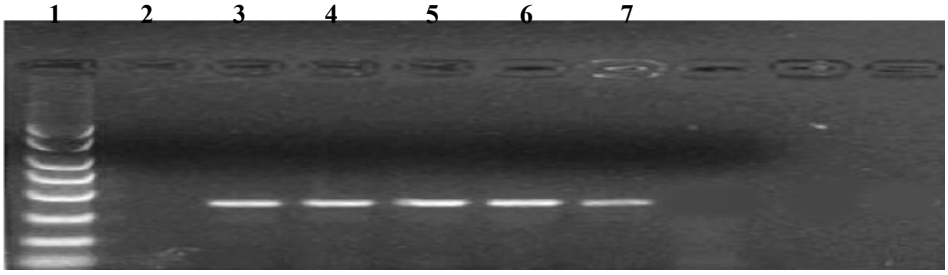

Fig. (2): Sequencing of a part of S1 gene. Lane 1 marker. Lane 2 negative control. Lanes 3, 4, 5, 6 isolates code 4, 16, 18 and 23 gave positive results at 385 bp PCR product. And Lane 7 positive control.

Table (2): 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 L=Live I=Inactivated

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

Table (3): 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	a) Match for 100% with isolate CK/CH/LDL/011 isolated in China	DQ167130
				b) Match for 100% with isolate Q1 isolated in Singapore.	AF286302
16	Broiler, 24d	Yes (L)	a) Match for 98% with Connecticut strain.	a) Match for 96% with isolate Egypt/F/03.	DQ487085
			b) Match for 97% with M41 strain.	b) Match for 96% with isolate GX1-98 isolated in China.	A319302
18	Broiler, 32d	Yes (L)	No match with known type.	a) Match for 98% with isolate 720/99 isolated in Israel.	AY091552
				b) Match for 98% with isolate 885 isolated in Israel.	AY279533
23	Layer, 54w	Yes (L+I)	a) Match for 93% with 4/91.	a) Match for 98% with isolate FR-94047-94 isolated in France.	AJ618987
				b) Match for 96% with isolate Spain/92/51 isolated in Spain.	DQ064801

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

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

GAAAGGTTTATTGTTTATAGAGAAAGTAGTGTTAACACTACCTTAGTGTT
AACTAATTTTACTTTCTCAAATGTTAGTAACGCCCCTCCTAATACAGGTG
GTGTTCATAGTATTGTTTTACATCAAACACAAACAGCTCAGAGTGGTTAT
TATAATTTTAATTTCTCCTTTCTGAGTAGTTTCCGTTATGTAGAATCAGA
TTTTATGTATGGGTCATACCACCCAAAATGTTCATTTAGACTAGAAACTA
TTAATAATGGTTTGTGGTTTAATTCACTTTC

Fig. (3): IBV isolate (4) sequencing of 385 bp product of S1 gene. 100% homolgy to isolate CK/CH/LDL/011 isolated from China and Q1 strain from Singapore.

CAGAAGTTTATTGTCTATCGTGAAAATAGTATTAATACTACTCTTAAGTT
ACACAATTTCACTTTTCATAATGAGACTGGCGCCAACCCTAATCTTAGTG
GTGTTCAGAATATTCAAACTTACCAAACACAAACAGCTCAGAGTGGTTAT
TATAATTTTAATTTTTCCTTTCTGAGTGGTTTTGTTTATAAGGAGTCTAA
TTTTATGTATGGATCTTATCACCCAAGTTGTAATTTTAGACCAGAAACTA
TTAATAATGGCTTGTGGTTTAATTCACTTTC

Fig. (4): IBV isolate (16) sequencing of 385 bp product of S1 gene. 98% homolgy to Connecticut strain. 97% homolgy to M41 strain. 96% homolgy to isolate Egypt/F/03. 96% homolgy to isolate GX1-98 isolated in China.

GAAAAGTTTGTTGTGTATCGTGAAAATAGTTTTAATACTACTCAGGTTTT
AAATAATTTCACGTTTTATAATGAAAGTAATGCCCCTCCTAATGTTGGTG
GTGTTAATACTATTAATCTTTATCAAACACATACAGCTCAGAGTGGTTAT
TATAATTATAATTTATCATTCCTGAGTGGTTTTGTGTATAAAGCTTCTGA
TTTTATGTATGGATCTTATCACCCAAAGTGTGATTTTAGACCAGANACTA
TTAATAATGGTTTGTGGTTTAATTCTCTATN

Fig. (5): IBV-isolate (18) sequencing of 385 bp product of S1 gene. 98% homolgy to isolate 720/99 isolated in Israel. 98% homolgy to isolate 885 isolated in Israel.

GATAGGTTTATTGTATATCGAGAAAGTAGTATTAACACTACTTTAGAGTT
AACTAATTTTACTTTTACTAATGTAAGTAATGCTGCTCCTAACTCAGGTG
GCATTCAGACCTTTCAATTATATCAAACACACACCGCTCAGGATGGTTAT
TATAATTTTAATTTATCATTTCTGAGTGGGTTTGTGTATAAACCATCTGA
TTTTATGTATGGGTCTTACCACCCAAAGTGTAATTTTAGACCAGAGAATA
TTAATAATGACTTATGGTTTAATTCATTATC

Fig. (6): IBV isolate (23) sequencing of 385 bp product of S1 gene. 93% homolgy to 4/91. 98% homolgy to isolate FR-94047-94 isolated in France. 96% homolgy to Spain/92/51 isolated in Spain.

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 identifiying 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 transcripition - polymerase chain reaction (RT-PCR) amplification of S1 have become more commonly used because their short turn – around time and high degree of specificty (Kingham *et al.,* 2000).

Direct automated cycle sequencing (DACS) stratiges 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 similarities 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 Massachusettes 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). These findings 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 geograhic 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 Massachusettes 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 Massachusettes vaccine. Possibly, some immune responses, including non-specific ones, to the Massachusettes type vaccine would impede replication of variant type (Cavanagh *et al.,* 1999).

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مدى انتشار مرض الإلتهاب الشعبى المعدى فى بعض قطعان الدجاج

٢. التوصيف الجزيئى لفيروسات الإلتهاب الشعبى المعدى المعزولة من إصابات حقلية

أجريت الدراسة على عدد خمس وعشرين (٢٥) معزولة من فيروس الإلتهاب الشعبى المعدى من إجمالى ست وثلاثين (٣٦) مزرعة دجاج تسمين وبياض من ١٣ محافظة من محافظات مصر خلال عامين بدءاً من يناير ٢٠٠٣. كل المزارع التى تم منها العزل كانت محصنة ضد المرض بواسطة فيروس الإلتهاب الشعبى المعدى المضعف اتش ١٢٠ بالإضافة إلى فيروس الإلتهاب الشعبى المعدى الميت فى مزارع البياض. الفيروسات قيد الدراسة كانت قد خضعت مسبقاً للعزل على أجنة بيض الدجاج الخالى من المسببات المرضية وعلى خلايا الزرع الجنينى الكلوى. خضعت المعزولات لإختبار سلسلة تفاعلات البلمرة المتعددة العكسى بالإضافة إلى توصيف تتابع جزئى للسلسلة النيوكلوتيدية للجين (S1) لأربع معزولات (ثلاث منها من مزارع دجاج التسمين ومعزولة من مزرعة دجاج بياض والذى أظهر تشابه العترات المعزولة من فيروس الإلتهب الشعبى المعدى لعترات أسيا وأوروبا والولايات المتحدة الأمريكية والشرق الأوسط.