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COMPARISON BETWEEN HI AND ELISA IN DETECTING IMMUNE TITER FOLLOWING IBV VACCINATION

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ABSTRACT

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Serological assays can be used for evaluating immune response post vaccination, they can be also helpful in studying the status of maternally derived antibodies (MDA), and they can also give a diagnostic mirror for viral sero-epidemiology. In the present study comparison between hemagglutination inhibition test (HI) and Enzyme linked immunesorbant assay (ELISA) and their abilities to detect IBV antibodies at different circumstances (post vaccination, infection, and (MDA) was studied. HI test for IBV was performed against two distinct IBV serotypes namely (Mass- 41, 4/91). Since they are the major vaccines used commercially in the Egyptian market. ELISA was performed at two dilutions (1/100, 1/ 1000) which is nearly the reciprocal of dilution of (7 and 10) in HI test in a trial to set two points for comparing the obtained results From the two tests. ELISA test showed 100% sensitivity and specificity at dilution 1/100 and showed 80.96%, 95.5 % respectively at 1/1000 dilution. The sensitivity and specificity of HI test were 80.91%, 95% respectively when Mass-41 antigen was used and was 73.91 %, 62 % when 4/91 antigen was used .The difference in sensitivity and specificity with HI reflects it selectivity during serotyping and this picture will necessarily differ if samples were tested against other antigens like (D- 274, 1466,…..etc.,) this confirms our point of view for using HI in detecting immunity after IBV vaccination. It became obvious that ELISA result may be misleading as seen during studying MDA in sample (S-20), ELISA reading at 1/100 dilution was 12051±2018 with STDV(6384) and was 2406±754 with STDV(2385) at dilution 1/1000 their Conversion into two base log titer 1/10 will be 14.28±.317 and 14.56±.4 respectively on the other hand the HI titer was 4.5±.166 with STDV(0.5) when Mass – 41 antigen was used and it was 2.4±.476 with STDV (1.5) when antigen 4/91 was used, result of ELISA will be conflicting when devising a vaccination protocol for such flock.

Keywords: *IBV, HI, ELISA.*

INTRODUCTION

Infectious Bronchitis Virus (IBV) is a highly contagious acute viral disease of the upper respiratory tract of chickens, it can also replicate in epithelial tissues of kidneys, gonads and oviduct of chickens causing their pathology and affecting the performance Lee *et al.* (2004).

IBV contains four structural proteins. The spike (S), and membrane (M) glycoproteins, a small membrane protein (E), and the internal nucleo-capsid protein (N) Spaan *et al.* (1988). The S protein is comprised of two subunits, S1, which forms the globular head of the spike protein, and S2, which anchors the S protein in the membrane. The S1 glycoprotein is known to induce the production of neutralizing and hemagglutination inhibition (HI) antibodies Ignjatovic and Galli (1995). The N protein is involved in cell-mediated immune responses while the M protein of IBV is not recognized as an immunogenic protein Seo *et al.* (1997).

Prevention of IB is achieved mainly through vaccination. Although in most cases IBV strains within a geographic region are distinct as mentioned by Callison *et al.* (2001), Gelb *et al.* (2005), Ignjatovic *et al.* (2006). Because IBV undergoes frequent changes in the viral genome, mainly in the S1 gene which result in point mutations promoting the emergence of new antigenic variants Bochkov *et al.* (2007), Ammayappan *et al.* (2008) and Lee *et al.* (2008). The multiple IBV serotypes and its antigenic variation adds complexity to the proper selection of vaccination protocol and proper selection of serologic method to analyses the test results Jackwood and De Wit (2013).

Vaccine strains should be selected to represent the antigenic spectrum of isolates in a particular region, because attenuated vaccines are known to have a limited range of protection, confined in many cases to homologous strains, rendering vaccination partially successful Lin *et al.* (2005). The use of heterologous vaccine strains, either simultaneouor sequentially, has broadened the protection spectrum in some cases Cook *et al.* (1999), but it is difficult to predict which combinations may confer the best protection. On the other hand, more virulent vaccine strains may have a broader range of protection, but their use is not recommended to avoid the risk of a disease outbreak Darbyshire (1985).

In the present study we were focusing on the value of HI test in detecting antibodies against IBV, since it can give an individual outlook for the immune titer for each vaccine used when distinct IB viral antigens are used, this enables corrective action whenever a problem is detected. On the contrary to ELISA which is giving one total outlook for IBV immune titer post vaccination.

MATERIALS

1 -Serum samples

Table (1) shows the vaccination history, source and number of the collected serum samples.

Table 1: Vaccination history of samples under investigation.

Lab. Ref. No.	No. of samples	Breed	Age at sample collection	Vaccination history	
				Age In days	Vaccines
S - 8	10	Bro.	24 D	**1 D**	**HB1+IBV (H120)**
				9 D	**IBV - Ma 5**
				14 D	**IBV - 4/91**
S - 14	22	Lay.	182 D	**1 D**	**IB Primer(H120+D-274)**
				9 D	**IBV - 4/91**
				21 D	**IBV – Ma 5**
				35 D	**Triple inact.(NDV,EDS76r,IBV)**
				42 D	**IBV – H 120**
				63 D	**IBV – Ma 5**
				100 D	**IBV- H120**
				110 D	**Triple inact.(NDV,EDS76,IBV)**
S - 15	18	Lay.	280 D	**1 D**	**IBV- H120**
				8 D	**Triple inact.(NDV,EDS76,IBV)**
				15 D	**IBV - 4/91**
				40 D	**IBV – Ma 5**
				80 D	**IBV - 4/91**
				102 D	**Triple inact.(NDV,EDS76,IBV)**
S – 16	4	Bro.	32 D	**1 D**	**IB Primer(H120+D-274)**
				14 D	**IB Primer(H120+D-274)**
S – 18	4	Bro.	32 D	**No data**	**Randum**
S – 19	10	Lay.	245 D	**6 D**	**HB1+IBV(H 120)**
				13 D	**Triple inact.(NDV,EDS76,IBV)**
S - 20	10	Bro.	1 D	**---**	**MDA**
S - 22	6	Lay.	245 D	**1 D**	**H120**
				8 D	**Triple inact.(NDV,EDS76,IBV)**
				15 D	**IBV - 4/91**
				40 D	**IBV – Ma 5**
				80 D	**IBV - 4/91**
				102 D	**Triple inact.(NDV,EDS76,IBV)**
S - 23	8	Bro.	24 D	**1 D**	**IB Primer(H120+D-274)**
				7 D	**HB1+IBV**
Total				**92 sample**	

Lay = layers D=day Bro = broilers MDA=maternally derived antibodies

2 - Chicken RBCS
Chicken RBCS were obtained from three 28 day old specific antibody negative chicken (SAN) raised for this purpose.

3 - Saline
Sodium chloride 0.9% **(ADWIC) ®, Sterile** Pyrogen free.

4 - ELISA kit.
Commercial IBV ELISA kit (Proflock; Synbiotics Corporation). Lot.1202487

5 - IB viral antigen
IB viral antigens for HI test were obtained from GD Holland, {(Mass- 41) lot 11531-010811 exp. 8/2021, (4/91) lot 09601-020209 exp.8/2019.

6 - Negative serum
Sera from day old SPF chicks were used.

METHODS

Haemagglutination inhibition test (HI).
HI tests, using IBV antigens (Mass-41 and 4/91) were performed as described by **Villegas (1991), Villegas (2006).** Serum dilutions ranged from 1:2 to 1:1024. All HI titers were expressed as Iog2 of the reciprocal of the highest serum dilution showing complete HI.

ELISA test
Serum samples were assayed in the commercial IBV ELISA systems 1:100 and 1:1000 dilutions, respectively, according to the manufacturer's instructions. Serum-to positive ratios (SP-ratios) were calculated, using the SP ratio formula:

From these SP-ratios, titers expressed as Iog2 values, were calculated using identical regression formulas (Synbiotics software) for both tests. Also used were the positive-negative SP cut-off values of 0.200 Synbiotics IBV ELISA according to the manufacturer recommendation.

$$\frac{\text{OD sample — OD negative control}}{\text{OD positive control — OD negative control}}$$

Statistical analysis.
Data were statistically analyzed as described by Snedecor and Cochran (1967). Using SPSS.16 computer program, value was used to determine significance.

RESULT

Results of the present work are illustrated in tables (2-7).

Table 2: The Geometric mean titer (GMT) of ELISA titer at dilution 1/100, and their conversion into two base logarithmic titer 1/10.

GMT of Elisa titer at dilution 1/100									Converting GMT of Elisa titer at dilution 1/100 multiplied *10 into two base log.titer 1/10 (Brugh 1978)								
A - Portion									*B-Portion*								
S - 8	S -14	S -15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23	S - 8	S -14	S-15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23
20356	5788	19443	5960	1355	15032	13455	9684	26322	15.3	13.5	15.2	13.5	11.4	14.8	14.7	14.2	15.7
8642	16496	11677	10619	6865	17853	4213	3785	22288	14	15	14.5	14.3	13.7	15.1	13.1	12.8	15.4
7497	17169	18039	5041	8722	19884	17483	8984	17853	13.8	15.1	15.1	13.4	14	15.2	15.1	14.2	15.1
11099	13717	22869	1269	20861	22694	11275	4441	7648	14.4	14.7	15.4	11.4	15.3	15.4	14.5	16	13.9
9746	22869	23132			16702	16857	4612	15681	14.2	15.4	15.5			15	15	13.1	14.9
17747	20189	21455			23249	13861	6415	16137	15.1	15.3	15.4			15.5	14.7	13.6	14.9
8286	22869	17481			13813	11727		1688	14	15.4	15.1			14.7	14.5		11.7
76686	22345	22201			7478	2330		9935	15.9	15.4	15.5			13.8	12.2		10.9
10147	20664	14126			3901	6327			14.3	15.3	14.7			12.9	13.6		
3974	20078	17169			8783	22985			12.9	15.2	15			14.1	15.4		
	21427	21798								15.3	15.4						
	21002	22172								15.3	15.4						
	16086	20468								14.9	15.3						
	21569	21970								15.4	15.4						
	19279	22259								15.2	15.4						
	20636	17510								15.3	15.1						
	23220	16444								15.5	15						
	21256	17143								15.3	15						
	14077									15.3							
	21856									14.7							
	20973									15.4							
	21455									15.3							
10	22	18	4	4	10	10	6	8	10	22	18	4	4	10	10	6	8

Table 3: The Geometric mean titer (GMT) of ELISA titer at dilution 1/1000, and their conversion into two base logarithmic titer 1/10.

GMT of Elisa titer at dilution 1/1000									Converting GMT of Elisa titer at dilution 1/1000 multiplied *100 into two base log. titer 1/10 (Brugh 1978)								
A-Portion									*B*-Portion								
S - 8	S -14	S-15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23	S - 8	S -14	S-15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23
8041	4408	8596	280	0	5664	2155	237	4252	15.9	15.9	16.9	12.4	0	16	15.3	15.5	16
212	4836	2760	1489	1894	5912	525	405	9322	12.1	15.9	12.4	14.8	15.2	16	13.3	16	18
237	3577	5028	276	4728	6866	4660	819	7631	12.2	15.9	15.9	12.4	15.9	16	13.1	14	18
1272	4501	12024	331	7892	1077	1077	289	1584	14.5	15.9	18	12.7	17.9	14.3	14.3	12.5	14.9
805	2378	14212			0	1999	0	3723	13.9	15.5	18			0	15.2	0	15.9
1847	3784	7810			5406	2482	675	4986	15.2	15.9	17			16	15.6	13.7	16
1177	13718	3689			4890	1866		930	14.5	16	16			16	15.1		14.1
1413	8191	10072			7696	469		1305	14.8	17	17			17	13.1		14.6
233	5014	2814			2870	644			12.1	15.9	15.7			15.8	13.6		
0	5693	2673			2009	8191			0	15.9	15.7			15.2	17		
	5477	9358								15.9	17						
	11504	12160								17	18						
	1980	7975								15.3	17						
	1838	12770								15.1	18						
	2524	15821								15.6	18						
	8630	9393								16	17						
	17925	3577								18	16						
	9517	7054								17	17						
	2970									17							
	2429									15.8							
	5222									15.5							
	8325									16							

Table 4: Haemagglutination inhibition (HI) titer of examined samples using Mass-41 and 4/91 IBV antigens

Titer values	HI Mass-41										HI- 4/91									
	S - 8	S -14	S-15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23	total	S - 8	S -14	S-15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23	total
	Number of values										Number of values									
0				**1**					**3**	**4**				**4**	**1**	**10**	**1**		**8**	**24**
1									**5**	**5**					**1**		**2**			**3**
2	**1**				**1**	**2**				**4**	**5**				**1**		**2**			**8**
3	**2**				**2**	**2**				**6**					**1**		**3**			**4**
4	**1**	**5**			**1**	**2**	**5**			**14**	**5**	**4**					**1**	**3**		**13**
5	**3**	**8**		**1**		**3**	**5**			**20**		**8**					**1**	**3**		**12**
6	**2**	**5**	**1**			**1**				**9**		**6**	**9**							**15**
7	**1**	**2**	**5**	**2**						**10**		**3**								**3**
8		**2**	**12**							**14**		**1**	**9**							**10**
9																				
10								**6**		**6**										
total	**10**	**22**	**18**	**4**	**4**	**10**	**10**	**6**	**8**		**10**	**22**	**18**	**4**	**4**	**10**	**10**	**6**	**8**	
	Cut off values																			

Table 5: The Geometric mean titer (GMT) of ELISA titer at dilution 1/100, 1/1000 and their conversion into two base logarithmic titer 1/10.

	S - 8	S -14	S -15	S - 16	S - 18	S - 19	S - 20	S - 22	S - 23
A	17418±6759	19319±869	19279±772	5722±1922	9450±4112	14938±2060	12051±2018	6320±1021	14694±2826
STDV	21376.7	4078.2	3277.1	3844.05	8225	6514.3	6384	2501.8	7994.3
B	14.39±.2	15.14±.09	15.1±.06	13.15±.6	13.6±.8	14.65±.25	14.28±.317	13.9±.465	14.1±.6
STDV	.851	.427	.278	1.23	1.6	.81	1.004	1.139	1.7
C	1523.7±750*	6110±883	8210±980	594±298*	3628.5±1721	4239±818	2406±754	404±122	4216±1075**
STDV	2372.4	4144.8	4160.6	597.1	3442.9	2588	2385	299.9	3041.8
D	12.52±1.45	16.09±.14	16.7±.319	13.07±.57	12.25±4.1	14.23±1.59	14.56±.4	11.9±2.44	15.9±.51*
STDV	4.6	.686	1.36	1.15	8.2	5.04	1.29	5.98	1.45
E	4.7±.5	5.45±.26	7.61±	4.75±1.6	3±.405	3.9±.433	4.5±.166	10	0.63±.18
STDV	1.766	1.22	0.6	3.3	0.81	1.37	0.5	0	0.517
F	3±.33	5.5±.23	7±1.02	0	1.5±.64	0	2.4±.476	4.5±.22	0
STDV	1.05	1.1	1.02	0	1.29	0	1.5	0.54	0

*A=GMT of Elisa titer at dilution 1/100 *=0.05 **=0.01.*
B=Converting GMT of Elisa titer at dilution 1/100 into two base logarithmic titer 1/10 (Brugh 1978).
C=GMT of Elisa titer at dilution 1/1000 STDV. =standard deviation
D=Converting GMT of Elisa titer at dilution 1/1000 into two base logarithmic titer 1/10 (Brugh 1978).
E= Mean HI -Mass 41.
F= Mean HI -4/91

Table 6: Shows the sensitivity % and specificity % of applied serological tests.

	ELISA at dilution 1/100	ELISA at dilution 1/1000	HI using mass-41	HI using 4/91
*** Sensitivity %**	100%	80.96%	80.91%	73.91%
****Specificity %**	100%	95.5%	85%	62%

* Sensitivity =% of positives / tested samples. **specificity = % of negatives / tested samples

Table 7: Shows the correlation between number and varieties of given vaccines, beside intervals up till sample collection and the recorded serological values with different serological tests.

	Number and varieties of vaccinal strains given for examined flocks						Time elapsed from vaccination till sampling	HI titers		ELISA two base log.titer 1/10
	Classical vaccinal strains			Triple inactivated vaccine	Variant vaccinal strains					
	H 120	Ma 5	Mass-41		4/91	D 274		Mass-41	4/91	1/100 dilution
S - 8	I	I	-	-	I	-	**10 days**	**4.7**	**3**	**14.39**
S -14	III	II	-	II	I	I	**72 days**	**5.45**	**5.5**	**15.14**
S - 15	I	I	-	II	II	-	**178 days**	**7.61**	**7**	**15.1**
S - 16	II	-	-	-	-	II	**17 day**	**4.75**	**0**	**13.15**
S - 18				**Random**				**3**	**1.5**	**13.6**
S - 19	I	-	-	I	-	-	**232 days**	**3.9**	**0**	**14.65**
S - 20				**MDA**				**4.5**	**2.4**	**14.28**
S -22	I	I	-	II	II	-	**143 day**	**10**	**4.5**	**13.9**
S - 23	II	-	-	-	-	-	**17 day**	**0.63**	**0**	**14.1**

I = administered once II = administered twice III = administered three times - = non

DISCUSSION

Prevention of IB relies mainly on vaccination, however it does not confer full protection against the heterologous IB variant strains. To devise a successful vaccination protocol against IBV it is necessary to isolate the circulating viruses in the locality and study their relatedness and cross protection Callison *et al.* (2001). The various viral strains of IB can be classified according to serotype, genotype and protectotype. using virus neutralization, or by genotyping, using RT PCR together with sequencing or a second specific-primer PCR, or, more rarely, using restriction fragment length polymorphism (RFLP) Worthington *et al.* (2008), this may not be feasible in developing countries and it is time consuming. Other workers such as Cook *et al.* (1999) though of combing classical and variant vaccines to widen the protection against IBV but this was not completely successful due to the large number of IBV serotypes and the lack of complete cross protection.

We thought that it might be helpful to use HI for the evaluation of the immune titer for two most prevalent vaccine strains in the Egyptian market, since this test is efficient in serotyping which will helpful in studying sero-conversion post vaccination with these strains, this procedure will allow a corrective procedure when vaccinal failure is detected, and we also suggested rules for interpretation of the HI and ELISA results.

The multiple IBV serotypes and its antigenic variation, adds complexity to the selection of an appropriate serologic method and to the analysis of test results. All IBV serotypes would seem to have common epitopes (group-specific antigens), which is given the moderately high amino acid sequence identity within the N and M proteins and the S2 part of the spike protein. IBVs also induce type-specific antibodies, determined by epitopes of the S1 protein Cavanagh and Gelb (2008). Jackwood and De Wit (2013).

The advantage of serological studies based on tests such as (HI and ELISA) is the historical aspect that they bring to the clinical case, since their results can measures the reaction of the bird to the disease causing agent over a period of time, whereas direct diagnostics such as (PCR) requires the presence of the virus at the very moment of sampling Auvigne *et al.* (2013).

ELISA testing is unable to differentiate the different infectious bronchitis serotypes, as this method involves antibody binding to group-specific as well as type- specific antigens Mockett and Darbyshire (1981). Macnaughton *et al.* (1981). The antibody response assessed by HI and VN, may be used as an aid to the diagnosis of the predominant serotype present. Dhinakar and Jones (1997), Mockett and Darbyshire (1981).

In poultry, high specificity of serological tests is more important than high sensitivity, since low sensitivity can be compensated for, by using a higher number of blood samples. The sensitivity could be calculated as the percentages of positives while the specificity could be calculated as the percentage of negatives in the tested samples De Wit *et al.* (1997), De Wit (2000), Auvigne *et al.* (2013). In the present study the sensitivity of ELISA at dilution 1/ 100 was 100 %, and this percent was 80.96% when the dilution was 1/ 1000, comparatively the HI test performed using Mass-41 antigen at cut off value 2 revealed that 80.92 % of the examined samples were positive for IBV antibodies and this percent was 73.91 % when the 4/ 91 IBV antigen was used. The cut off value in the present study was set as 2^2 (table -4) on the contrary for the previous work of Alexander and Chettle (1977), De wit *et al.* (1997) because Villegas (1991), Villegas (2006) were using 8 HA units ,while Alexander and Chettle (1977), De wit *et al.* (1997) used 4 HA units of IBV antigen for the test, as for the specificity in the present study the percent of negative reactors in the examined samples using ELISA were (0/92) at dilution 1/100 and was (4/92) i.e. 4.3% when ELISA was performed at dilution 1/1000, and when HI was performed it was (14/92) i.e. 15.2% using Mass-41 antigen, and (35/92) i.e. 38% with 4/91 antigen. De wit *et al.* (1997) found that ELISA is moderately sensitive but highly specific when examining sera from non-vaccinated birds ,and it is sensitive with variable specificity when examined sera were from vaccinated birds., as for HI test the results will depend on the cut off values and they recorded a specificity of 55 - 100% and wide variation in sensitivity. These result fortifies our findings, the wide variation in sensitivity is result of using distinct antigen i.e. (4/91 or Mass-41).

ELISA showed 100% sensitivity and specificity at dilution 1/100 and showed 80.96%, 95.5 % respectively at 1/1000 dilution. The sensitivity and specificity of HI were 80.91%, 95% respectively when Mass-41 antigen was used and was 73.91 %, 62 % when 4/91 antigen was used. The difference in sensitivity and specificity with HI reflects it selectivity during serotyping and this picture will necessarily differ if samples were tested against other antigens like (D- 274, 1466,…..etc.,) this confirms our point of view for using HI in detecting immunity after IBV vaccination.

It became obvious that ELISA result may be misleading as seen during studying MDA in sample (S-20), ELISA reading at 1/100 dilution was 12051±2018 with STDV (6384) and was 2406±754 with STDV(2385) at dilution 1/1000 their Conversion

into two base log titer 1/10 will be 14.28±.317 and 14.56±.4 respectively on the other hand the HI titer was 4.5±.166 with STDV(0.5) when Mass – 41 antigen was used and it was 2.4±.476 with STDV (1.5) when antigen 4/91 was used, result of ELISA will be conflicting when devising a vaccination protocol for such flock.

The rational for ELISA at dilution 1/1000 is the high immune titers observed when dilution was 1/100, and because that 4 out of 92 examined sample were negative (table -3) although 1:1000 in ELISA is very near for 1:1024 (10^{th} dilution of HI starting 1:2) at which 6 samples out of 92 were only positive and the remaining 84 samples ended before the 9^{th} dilution, this had led us to carry on ELISA at dilution 1: 100 which is very near for 1:128 (7^{th} dilution of HI starting 1:2) all examined samples were positive for ELISA and HI using Mass-41 IBV antigen but a different picture was observed when HI using 4/91 IBV antigen this confirms the hypothesis that with HI you can observe a distinctive pictures for each vaccinal strain used .although examined samples were (100%) positive for ELISA and HI using Mass-41 IBV antigen at dilution 1:100 and were (13/93) i.e. 14.13% when HI was performed using 4/91 antigen, and this confirms our point.

The ELISA technique is a sensitive serological method gives earlier reactions and higher antibody titers than other tests Mockett and Darbyshire (1981). Commercial ELISA kits used for monitoring serum antibody responses against IBV are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges but it lacks strain specificity Karaca and Naqi. (1993), De Wit *et al.* (1997) commercially available. ELISAs detects IBV antibodies within one week of infection, earlier than by HI or VN tests Marquardt *et al.* (1981), Mockett and Darbyshire (1981), De Wit *et al.* (1997), De Wit (2000).

HI should be used for identifying serotype-specific responses to vaccination and field challenges in young growing chickens. Because with multiple infections and vaccinations, the sera of breeders and layers will be cross-reactive thus its results cannot be used with a high degree of confidence De Wit *et al.* (1998)., but the low cost, simple test equipment and speed of the HI test makes it a very useful procedure for routine diagnosis; the limitations must simply be borne in mind, and alternative analytical techniques should be at hand when doubt arises De Wit *et al.* (1997).

Standard protocol for HI for IBV has been described Villegas (1991), Villegas (2006) with minor changes from the protocol of Alexander and chettle (1977), the test procedure is based on that standard strains and isolates of IBV will agglutinate chicken red blood cells RBCs after neuraminidase treatment Ruano *et al.* (2000), Schultze *et al.* (1992). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. In the present study sera were analyzed by HI for two serotypes (Mass41and 4/91). The serum samples underwent serial halving dilution and were mixed with an equivalent amount of antigen. After incubation (30 min at 20°C), washed chicken red blood cells (WCRBCS) from SAN chickens, prepared with an anticoagulant, were added. After incubation (40mn at 20°C), the results were read by inclination of the plaques and observation of the HI. The antibody titer of the serum sample, expressed in log2, corresponds to the highest serum dilution leading to complete inhibition. The highest tested titer is 10, the well 11 was left for virus control and well 12 was left for (WCRBCS) control. The validity of results was ascertained by using a negative serum control sample that should not have a titer above or equal to 2 log2.

Haemagglutinating antigen for the HI test could be prepared from chorioallantoic fluid harvested from IBV-inoculated embryonated chicken eggs. Neuraminidase type V in PBS (pH7.2 at 1, 0 units/ml final concentration is used to treat IBV for 30 min at 37 C) Ruano *et al.* (2000). Antigen titration is performed in standard U-bottom 96-well microtiter plates using (WCRBCS) close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc. gives more accurate initial titration. Treatment of IBV with bacterial phospholipase C was initially thought to enable the virus to agglutinate (WCRBCS) Alexander and Chettle (1977), King and Hopkins (1984). However, HA antigens produced using highly purified phospholipase C preparations often had considerably lower titers than those produced using unpurified phospholipase C preparations. Subsequent studies Ruano *et al.* (2000). Shultze *et al.* (1992) determined that treatment of IBV with purified neuraminidase preparations consistently produced high-titer HA antigens. These findings suggested that the unpurified phospholipase C preparations were contaminated with neuraminidase, this comparison will be the subject of our investigation during the intended antigen preparation trials.

Auvigne *et al.* (2013) used typology of the flock profiles using clustering methods. This allows grouping the flocks according to their resemblance without posing any prior hypothesis other than those used for the general study design (choice of flocks, age at sampling, sampling and testing). As a first step, a principal components analysis (PCA) was carried out to allow studying the overall variability of the dataset and creating new uncorrelated variables, summarizing information that may have a biological sense and that may contribute to differentiate groups. The study of the variability percentage explained by each of this new variables and their biological

signification allow determining which of these new variables have to be retained for the rest of the analysis. As a second step, ascending Hierarchical Clustering (HC) was performed. For calculation of distances between individuals and then assembling them in groups according to proximity. The variables used in the HC to calculate the distances are the coordinates of the flocks on the retained axes by the PCA. The HC leads to a dendrogram used to define the optimal cluster number. This choice depends on the clearness of the separation between the dendrogram branches and on the number of flocks in each group. As a third step, the means of the new variables created at the PCA step are calculated for each of the groups and, using the k-means method, each flock is reassigned to the group with the nearest mean. The representation of these groups on the PCA axes allows determining the discriminatory axes between groups. If certain axes turn out to be non-discriminatory, a new iteration of the analysis is performed. Finally, as a fourth step, the distribution characteristics of the 6 mean serological titers within each group are described and the biological meaning of these groups is investigated. Auvigne *et al.* (2013) mentioned the typological analysis allows interpretation of the results in spite of the existence of cross reactions. However, this method requires certain choices (number of axes, number of clusters) that have an impact on the final result, and it is not an absolute classification. However, the fact that the obtained classification is coherent with the data of experimental infections, even though the bibliographical data were not used for the classification, is in favor of its relevance. In the present study we were not able to use hierarchical clustering because setting a cut off values will not allow to consider values below this cut off .In the present study we used the EXCEL sheets to calculate what is presented in (table -5).

ELISA titers as seen in (table 2,3) couldn't be evaluated as it is, and It was also noticed the standard error of the values was very high ranging from (4 to 43%) which is a very high values indicative of nonhomogeneous titers Kurian *et al.* (2012) examined heat treatment, repetitive freezing and thawing and three levels of severity of hemolysis as a sample mishandling treatments simulated different conditions that might occur during routine blood collection, transport or storage in a clinical practice setting. Each mishandling treatment was experimentally applied under laboratory conditions and then samples were assayed for antibodies against IBV, AEV and CAV using commercial ELISA kits. And found that severe hemolysis had the most consistent detrimental effect on ELISA performance, producing results that were significantly different from the reference standard in all three ELISAs, although the direction of the effect varied (less positive for the IBV and CAV assays; more positive for the AEV assay). Moderate levels of hemolysis had a similar, but less consistent, effect to that of severe hemolysis, producing results that were significantly different from the reference standard only for the IBV (less positive) and AEV (more positive) ELISAs. Repetitive freeze–thawing also produced a significant effect on ELISA results for IBV (less positive) and AEV (more positive). The IBV ELISA appeared to be most susceptible to the effects of serum maltreatment. The findings from this study suggest that unpredictable variation in the results of ELISAs can occur due to different sample mishandling treatments.

In the present study we converted the obtained ELISA results at dilution 1/100 in (table-2) and that at dilution 1/1000 (table -3) into its two base logarithmic titer 1/10 (Brugh 1978), to obtain a numerical values that well facilitate the comparison between the two tests as performed by De Wit *et al.* (1997) After converting the GMT of ELISA titer 1/100 multiplied by10 into its base log titer 1/10 (Brugh 1978), you can observe that the figure 15.4 will represent the ELISA reading of (20973-22869) (table -2), this numeric values were very high compared to that of HI at dilution 7 or 10 which were zero in most examined samples.

In the present study serum samples submitted for investigation were collected from different flocks receiving different vaccination programs (table 1), the time interval from the last vaccine used until sample collection ranged from 10 to 232 day post vaccinations (Table-6), the mean two base log titer of ELISA at dilution 1/100 was ranging from (13.16 - 15.14) despite of the number of vaccine administration which ranged from (2 up to 9 times including inactivated vaccine) (table -6). It is also apparent that this high titers was observed in samples (S – 16,19 and 23) these samples were from flocks that did not receive any variant vaccines, this apparent high titer masks the fact that birds are at risk if challenged with a variant. Comparatively HI titers observed were zero in sample (S- 16, 19 and 23) since their flock did not receive any vaccine of such strains so, although ELISA gave a sensitivity percent of 100%, 80.96% at the examined dilution (1/100, 1/1000) respectively and it also gave a specificity (100%, 95.5) at the same dilutions, but this is not logic for the obtained results compared to HI results.

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المقارنة بين اختبار منع التلازن الدموي واختبار الاليزا في الكشف عن الاستجابة المناعية بعد التحصين ضد فيروس الالتهاب الشعبي

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في دراستنا اردنا الوقوف علي قيمة اختبار منع التلازن الدموي عند دراسة الاستجابة المناعية بعد التحصين ضد فيروسات مرض الالتهاب الشعبي و ذلك لقدرته التفريقية بين مختلف عترات الالتهاب الشعبي. حيث انه بعد معالجة عترات فيروس الالتهاب الشعبي بإنزيم النيورامينيديز او الفوسفوليبيز يكتسب فيروس الالتهاب الشعبي القدرة علي تلزين كرات الدم الحمراء. وبذلك يتيح اختبار منع التلازن الدموي دراسة الاستجابة المناعية لكل عترة تحصين فيروسية علي حدة مما يتيح التدخل لوضع حلول عند اكتشاف فشل في التحصين لإي عترة تحصين سبق استعمالها وذلك في محاولة لخدمة صناعة الانتاج الداجني في تتبع اي فشل في عملية التحصين. وكذلك لما هو ثابت علمياً من ان اختبار الاليزا يعطي صورة واحدة اجمالية للمناعة ضد فيروس الالتهاب الشعبي مما يؤدي بالتبعية الي خلل في تقييم الاستجابة المناعية للتحصينات ففي الوقت الذي يعطي اختبار الاليزا نتائج و قيم مناعية عالية تلاحظ لنا ان اختبار مانع التلازن يعطي قيم مناعية متدنية ضد عترة التحصين (mass-41) وضد العترة (4/91) وان هذا الاسلوب في تقييم المناعة يناسب ما هو معروف وثابت علمياً من وجود عترات كلاسيكية واخري مغايرة من فيروسات الالتهاب الشعبي وان التحصين بأياً منهم لا يكفي لصد العدوي بالاخر و هذا مما يعقد استراتيجيات المقاومة لهذا المرض الفيروسي. وهذة الفلسفة الاخيرة من فكر المقاومة تعود بنا مرة اخري الي قيمة العودة الي استعمال اختبار منع التلازن الدموي اكثر من مرة علي حدة بإستعمال عترات فيروسية ضد تلك اللقاحات المغايرة المستعملة حقلياً (D-274, QX, 4/91, 1466) وعترات التحصين الكلاسيكية (Mass TYPE). تحسب درجة دقة الاختبار بعدد الايجابي من جملة العينات المختبرة وتحسب درجة حساسيته بعدد السلبي من جملة العينات المختبرة وقد وجدنا ان دقة اختبار الاليزا ودرجة حساسيته كانت ١٠٠% عند اختبار التخفيف ١٠٠/١ وكانت ٨٠.٩٦% و ٩٥.٥ علي التوالي عندما تم الاختبار عند تخفيف ١٠٠٠/١ وقد كانت دقة الاختبار وحساسيته ٨٠.٩١ % و ٩٥% في اختبار مانع التلازن الدموي بإستعمال انتجين العترة الكلاسيكية (Mass-41) وكانا ٧٣.٩١% و ٦٢% في نفس الاختبار عند استعمال انتجين العترة المغايرة (٩١/٤) هذه النتائج تظهر القدرة الانتقائية في اختبار منع التلازن الدموي وقدرته علي اظهار صورة مناعية ضد كل عترة فيروسية علي حدة مختلفة عن الاخري في حين اظهراختبار الاليزا صورة مناعية جيدة هي اجمالية في عمومها ولكنها لم تكن كاشفة لكل حالة مناعية خاصة بكل عترة فيروسية استخدمت في عملية التحصين الوقائي. هذه الصورة سوف تختلف ايضاً بالتبعية حال استخدام انتجينات اخري مثل (D-274 or 1466….etc.,) وهذا يثبت صحة وجهة النظر التي اردنا بحثها. كانت لنا ايضاً بعض المآخذ علي اختبار الاليزا عند استخدامه في دراسة الاجسام المناعية الاميه لتقنين مواعيد التحصين وقد ظهر لنا هذا عند دراسة العينة (S-20) فقد وجدنا ان تتر الاليزا كان (٢٠١٨ ± ١٢٠٥١) بدرجة انحراف معياري (٦٣٨٤) عند التخفيف ١٠٠/١ وكانت النتائج (٧٥٤ ± ٢٤٠٦) بدرجة انحراف معياري (٢٣٨٥) عند التخفيف ١٠٠٠/١ وعند حساب اللوغاريتم الثنائي لهذة القيم عند التخفيف البادئ ١٠/١ كان المقابل (٣١٧. ±١٤.٢٨) و(٤. ±١٤.٥٦) وقد كان التتر المناعي للمناعة الامية بإستخدام اختبار منع التلازن الدموي (4. ± ٤.٥) بإنحراف معياري مقداره (٥.) عند استعمال انتجين العترة الكلاسيكية وقد كان التتر المناعي (١.٥± ٢.٤) بإنحراف معياري مقداره (١.٥) عند استعمال انتجين العترة المغايرة (٩١/٤) وبذلك نري ان اختبار الاليزا قد يؤدي الي خطأ في تحديد مواعيد التحصين.