Cytokine storm evaluation expression following experimental infection of native saso chickens with (IBDV.228 -E) at 7, 21 and 35 days of age

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Abstract: In the present study 160 day old native saso chicks were given the hot vaccinal strain (IBDV. 228-E) at 7, 21 and 35 days of age to study some of itspath -biological alteration. (Completeblood picture, bursal body weight index, Kidney functions, Cytokine storm components, pathological alterations in bursa, thymus and spleen, scoring of bursal lesion after virus administration, Beside studying the immunohistochemistry of bursae. Administration of the hot vaccinalstrain (IBDV.228- E) in native saso chicks having low MDA at the age 7,21 and 35 days was shocking since 7 days old chicks with low MDA did not show clinical disease when inoculated, and clinical disease was observed in most of the inoculated birds at 21 and 35 days of age. Morbidity was 60% while mortality was 12% when inoculation was made at 21 day of age. Morbidity was 80% and mortality was 8% when inoculation was made at 35 days of age. Typical PM lesion of IBD was recorded in sacrificed morbid or dead birds at 21 or 35 days of age. Administration of hot vaccinal strain (IBDV.228- E) in native as so chicks having low MDA at the age 7,21 and 35 days resulted in a bursal atrophy at the three age points evidenced by the results of BBI, pathological lesion scoring and immunohistochemistry examination. Assessment of the cytokine storm through ELISA estimation of the pro-inflammatories (IL-6, TNF, INFα $_{1/13}$) at the 2nd,4th, and 6th day following the (IBDV.228-E) administration at 7,21 or 35 day of age was done. Gotten results were utilized to interpret the recorded symptoms, mortality and immunosuppression.

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Key words: IBDV, ELISA, cytokine storm, intefron, interleukine -6, tumor necrosis factor,bursal lesion scoring, immunohistochemistry.

Abbreviations: **IBDV** =Infectious bursal disease virus**, TNF**=Tumor necrosis factor**, INF**=Interferon**, IL-6**=Interleukine-6 **BW** = body weight**; BRW** = bursal weight; **B:B**=bursal body weight ratio**; BBI**= bursal body weight index**; ELISA** = enzyme-linkedimmunosorbent assay**; BF**=Bursa of fabrecious**; AGPT**=Agar gel precipitation test; **PC** = post-challenge; **PI**=post infection: **IIP**=Indirect immunoperoxidase; **Ions**=inducible nitric oxide synthetase; **HRP**=horse raddish peroxidase; **PM**= post mortem; **AGPT**=Agar gel precipitation; **QAGPT**= Quantitative agar gel precipitation**; MDA**=Manternally derived antibodies; **GMT**= Geometric mean titer; **Std**=Standareddeviation; **S.E**=Standrederror; **V**=Variance; **SPF**=Specific pathogen free.

1. Introduction

Cytokines are proteins or peptides secreted by cells that play a key role in immune and inflammatory responses, through the activation and regulation of other cells and tissues. Epithelial cells may produce cytokines involved in the generation of inflammation, the so-called pro-inflammatory cytokines such as interleukin-6 or -8., whereas macrophages may produce both pro- inflammatory cytokines and cytokines involved in the activation and regulation of T helper lymphocytes in the development of an adaptive immune response. All cytokines act through receptors on the surface of the target cells, which may lead to the activation or down regulation of the cell's activity. Cytokines have been classified into a number of groups based on their activity and the cells they are produced by or act upon. These groups include interleukins (IL), interferons (IFN), tumour necrosis factors (TNF), transforming growth factors (TGF), migratory inhibitory factors and the smaller chemokines **Lunney (1998).**

Van den berg (2008) said that there is a growing evidence for a role of pro-inflammatory cytokines in the pathogenesis of IBD. He mentioned that during the

acute phase of IBD, there is a dramatic infiltration of T cells around the site of virus replication, in the bursa of Fabricius, spleen and caecal tonsils. The T lymphocytes does not support IBDV replication but they are activated and exhibit up-regulation of cytokine genes that has an effect on macrophage function with an exacerbated production of pro-mediators such as interferon (IFN α)1, tumor necrosis factor (TNF) α, interleukin(IL- 6 or IL-8). This cytokine storm induces a shock in the bird, which becomes prostrated and reluctant to move.

Kim et al. (2000) Mentioned that IBDV an avian B-lymphocyte tropic virus. It causes acute damage to the actively dividing immunoglobulin M-expressing (IgM^+) B cells {**Hirai et al., 1981, Nakai and Hirai 1981}.** The bursa is the principal reservoir of virus replication, and peak virus titers in the bursa can be detected between 3 to 5 days after IBDV infection **(Kaufer and Weiss 1980, Tanimura and Sharma1998).** Bursa of Fabricius is a unique, primary lymphoid organ in avian species, where B lymphocytes maturate and differentiate **Glick (1995).** The bursal follicles consist of B lymphocytes (85 to 95%), T cells (< 4%), and other non-lymphoid cells **(Ewert et al.1984, Chan etal.,1988, Palojoki etal.,1992, Khan and Hashimoto1996).** Productive IBDV replication in chicken bursae is often associated with necrosis, apoptosis of lymphoid cells, inflammatory change, atrophy, and hemorrhages **(Winterfield et al.1972, Hirai et al., 1981, Lam 1998, Tanimura and Sharma1998).** Chickens infected with IBDV experience suppression in both humoral immunity **(Giambrone et al.1977, Panigrahy et al., 1982, Dohms and Jaeger1988).** and cellular immunity **(Confer et al.1981, Panigrahy et al.1982, Thompson et al.,1997, Kim et al.,(1998).).** Humoral immunosuppression appears to be associated with IBDV-induced B-cell destruction, while the mechanism of cellular immunosuppression is largely elusive.

In IBDV-infected chickens, there was an increase in the numbers of intra-bursal T cells, while the bursae of uninfected chickens had very few resident T cells **(Khan and Hashimoto1996, Tanimura and Sharma1997, Kim et al., 1999 and Kim et al., 2000).** Bursal T cells were detected by immunohistochemistry at 1 day post-infection **(Tanimura and Sharma1997).** and persisted for several weeks **(Tanimura and Sharma 1997, Kim et al.,1999).** The infiltrating T cells were closely associated with the foci of viral antigen in bursal follicles. The majority of IBDV-induced bursal T cells were T-cell receptor 2-expressing (TCR^+) αβ T cells, and a few were ($TCR1^+$) γδT cells **(Tanimura and Sharma1997).**

In the present study we had rehashed the previous work of **Bayoumie et al., (2009)** and added a few research criteria to the previous work for further investigation of other aspects of the patho-biology met with IBDV, using hot (IBDV.228-E) strain.

2. Material and Methods

Materials

1-Chicks:

160 chicks hatched from fertile native saso eggs were used. The experimental design of the present work and the time marks for samplingis as described in table (1).

2- Hot IBDV vaccinal strain:

Hot vaccinal IBD isolate (228-E lotA094A1JO2, mfg. Date 11-2014. Exp. 11-2016. Intervet) was used. It was reconstituted in eye installation diluent and was passed through a 450 nm filter {Thermo scientific Nalgene syringe filter. Cat-no. 190-2545 (8-0404-40493)}. It was prepared to contain 10^3 VP / chick and was given for chicks at the time marks described in table (1).

3- Membrane filters:

450 nm Thermo scientific Nalgene syringe filter. Cat-no. 190-2545 (8-0404-40493).

4- EDTA.

Ready for use EDTA Obtained from Egyptian diagnostic media(EDM)., manf. 12/2015 exp. 12/2018 was used as an anticoagulant at a rate of 25 micron / 1 ml of blood**.**

5- Elisa kits for cytokine storm evaluation:

5.1-Human inter leukin-6 (IL-6), Catalog no.: E0079h, Exp.: 10/2016, Lot: 6D075C. Its homology to IL-6 chicken protein sequence is 32.787%personal communication with Eiaab via enny@eiaab.com.

5.2-Human Tumor necrosis factor (TNF), Catalog no.: E0133h, Exp.: 10/2016, Lot:6D075C.

5.3-Human interferon $\alpha_{1/13}$, Catalog no.: E0033h, Exp.: 10/2016Lot: 6D075C.

Its homology to (INF- $\alpha_{1/13}$) chicken protein sequence is 18.687% personal communication with Eiaab via enny@eiaab.com.

6- Ration

Starter pelleted ration "3000 k Cal ME energy, 21% protein and 2.88 % fat obtained from acommercial source was used, ration were used ad-libitum.

Table (1): Experimental design and sampling.												Pathology. and. immunohistochemisry
Age	IBDV. Challenge	Sampling Points	B:B. I	Bleeding time	Clotting time	Blood on edita	CBC	plasma				
								Cytokines			Kidney functions	
								IL-6	INF α 1/13	TNF		
7	+											
8												
9		+				+		+	+	+		
10												
11		+				+		+	+	+		
12												
13		+	+	+	+	+	+	+	+	+	+	+
21	+											
22												
23		+				+		+	+	+		
24												
25		+				+		+	+	+		
26												
27		+	+	+	+	+	+	+	+	+	+	+
35	+											
36												
37		+				+		+	+	+		
38												
39		+				+		+	+	+		
40												
41		+	+	+	+	+	+	+	+	+	+	+
+ = done												

Methods.

1-Hematological assay.

Bleeding time was performed as described by **Bigland (1964)**, in the meantime clotting time was performed as described by **Sankaranarayaan and Nambiar (1971)** and after that birds were sacrificed individually to obtain blood. Bit of the blood was collected on EDTA and another part was collected to obtain sera sample for determination of clinical chemistry parameters. The hematologicalexamine was performed utilizinga computerized animal hematology analyzer **(SYSMEX-XT-2000 iv).**

2-Clinico -chemical assay.

Serum uric acid and serum creatininewere done according to **Henry (1974)**. Cytokine storm assessment through examination of IL-6, $INF_{\alpha\ 1/13}$, TNF were assessed using ELISA kits at the time marks as described in table (1) and expressed as pg/ml.

3-Evaluation of immunosuppression.

Immunosuppresion following challenge with (IBDV. 228-E) was evaluated using the Bursal body weight index(BBI) as described by **Lucioand Hitchner (1979).**

4-Pathological examination.

Bursa, spleen and thymus specimens were collected after careful PM examination at the time marks described In (Table -1) then fixed in 10% formalin, and 5 u paraffin sectioned and stained with H&E according to **Suvarna et al (2013),** and examined with light microscope. Bursal lesionswere scored for the severity of histological lesions, based on the proportion of damaged follicles as described by **Lucioand Hitchner(1979)**and **Hassan et al., (2004).**

5-Immunohistochemistry examination.

Indirect immune-peroxidase(IIP) technique was utilized to detect IBD viral antigen in bursas obtained from infected and control chickens. The technique was applied on paraffin sections using positive slides according to the methods of **Suvarna et al (2013).** The primary IBD antibodies- Ig_g were developed in chicken and obtained from GD lab. Netherland., while the secondary antibody rabbit anti chicken horse raddish peroxidase (HRP) were from (KPL, USA).,

Substrate and chromagen DAB (Sigma) and counter stain Mayers Hematoxylin.

6-Statistical analysis.

Data were statistically analyzed as described by **Snedecor and Cochran (1967)** using SPSS.16 computer program, values were used to determine significance F-value was used to determine significance.

3. Result.

Results are as illustrated in Fig (1-24) and tables (2-7).

Fig.1: 21 day old native saso chick 36 hr following eye instillation of (IBDV. 228-E) showing depression, prostration, ruffled feather and reluctance to move. Picture was taken from Bayoumie et al (2016).

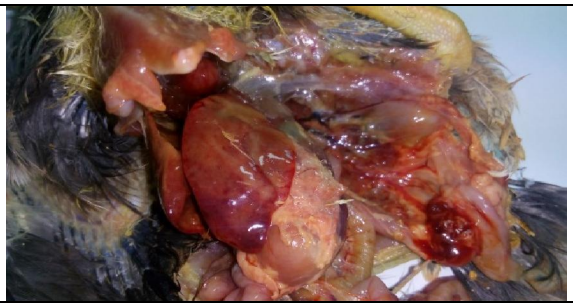


Fig.2: 21 day old native saso chick following eye installation of (IBDV. 228-E) showing hemorrhagic bursa besiderenal congestion.

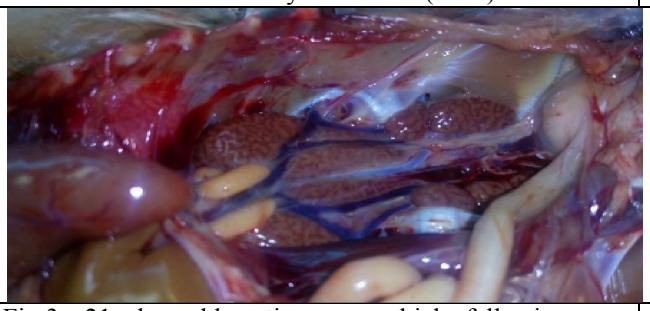


Fig.3: 21 day old native saso chick following eye installation of (IBDV. 228-E) showing inflamed bursa with a gelatinous transparent fluid and also showing congestion of kidney. Picture was taken from Bayoumie et al (2016).

Fig.4: 21 day old native saso chick following eye installation of (IBDV. 228-E) showing severe eroded hemorrhagicproventriculs.

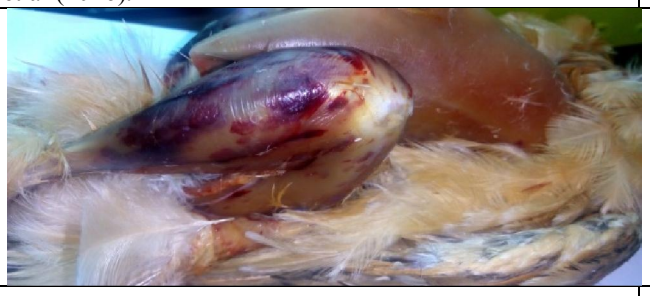


Fig.5: 21 day old native saso chicken 36 hr following eye instellation of IBDV. 228-E showing hemmorrages on the thigh muscles. Picture was taken from of Bayoumie et al (2016).

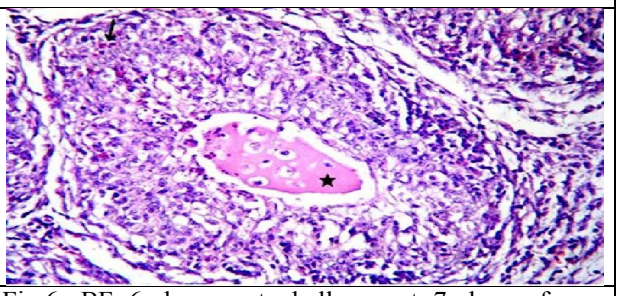


Fig.6: BF 6 days post challenge at 7 days of age with(IBDV. 228 -E) showing edema, necrotic debris in the center of few follicles (star) besideheterophilic infiltrations in cortex of depleted lymphoid follicles (arrow) H&E (X400) scored 3.

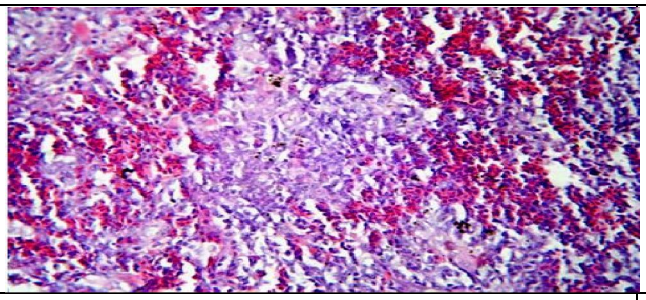


Fig.7: thymus 6 days post challenge at 7 days of age with (IBDV- 228 E) showing extensive hemorrhage in the medulla of thymic lobules H&E (X600).

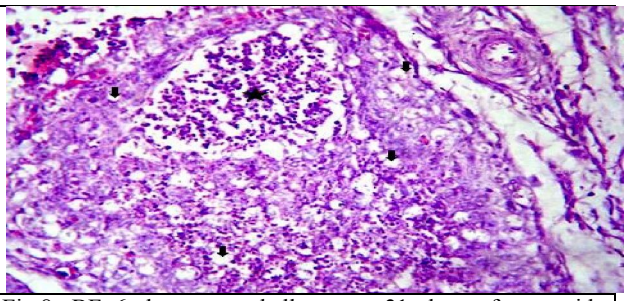


Fig.8: BF 6 days post challenge at 21 days of age with (IBDV- 228 E) showing focal necrotic changes (star) beside minute cystic spaces in both cortex and medulla of lymphoid follicles (arrows) H&E (X 400).

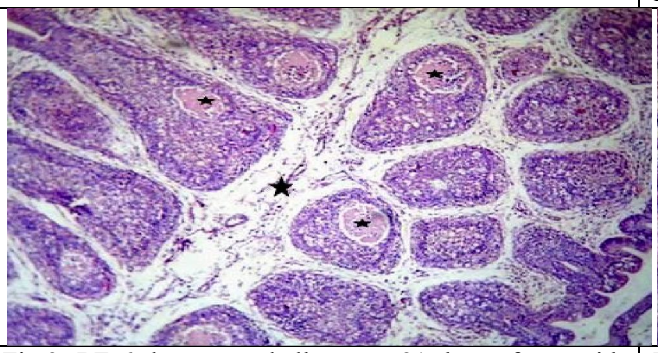


Fig.9: BF 6 days post challenge at 21 days of age with (IBDV- 228 E) showing edema in the majority of lymphoid follicles and in the inter follicular tissue (stars) H&E (X100) scored 5.

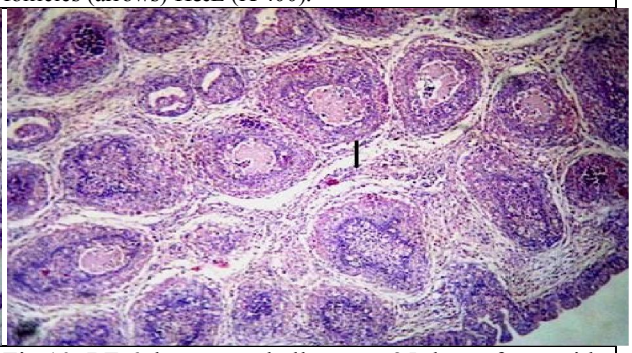


Fig.10: BF 6 days post challenge at 35 days of age with (IBDV- 228 E) showing extensive edema in depleted and atrophied lymphoid follicles beside fibroplasia in interfollicular tissue H&E (X100) scored 5.

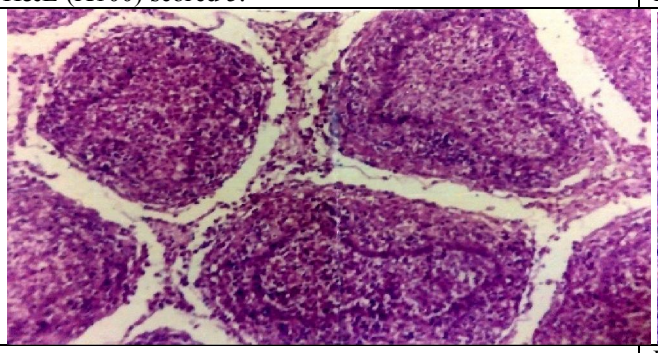


Fig.11: BF Showing a line of demarcation between cortex and medulla of lymphoid follicle following eye instellation of IBDV 228-EH&E (300X).

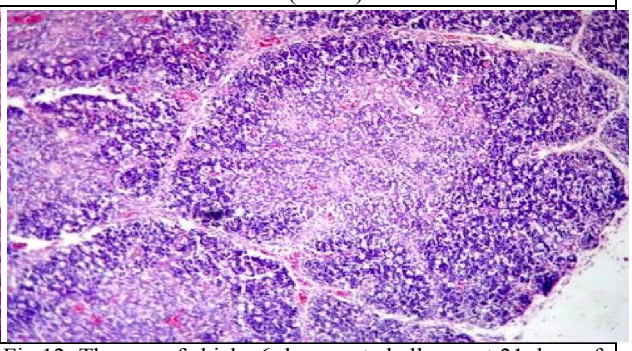


Fig.12: Thymus of chicks 6 days post challenge at 21 days of age with IBDV- 228 E) showing necrotic changes and minute cystic spaces in both cortex and medulla beside congestion and hemorrhage. H&E (X 100).

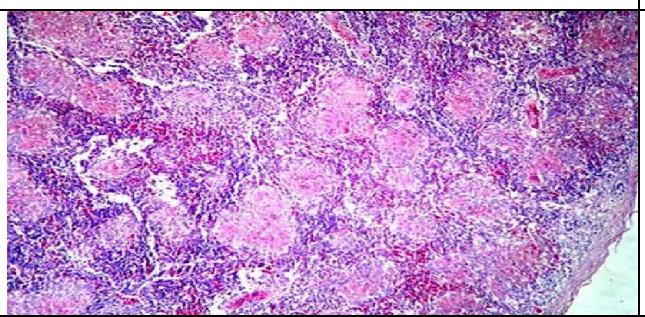


Fig.13: Spleen of chicks 6 days post challenge at 35 days of age with (IBDV- 228 E) showing multiple coagulative necrosis of splenic lymphoid follicles and hemorrhage. H&E (X200).

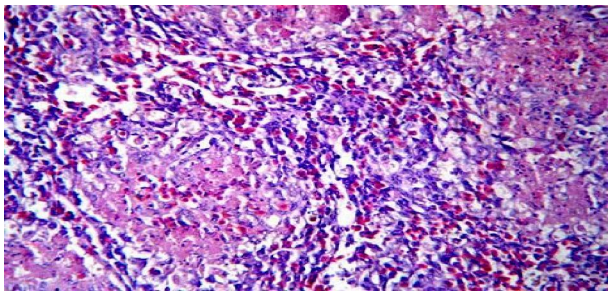


Fig.14: High magnification of Fig-13 shows extravasated erythrocytes in splenic tissue H&E (X600).

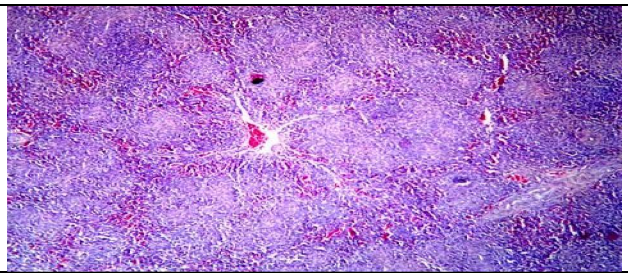


Fig.15: Chicken spleen at 13 days of age (control group) showing normal splenic tissue H&E (X 100).

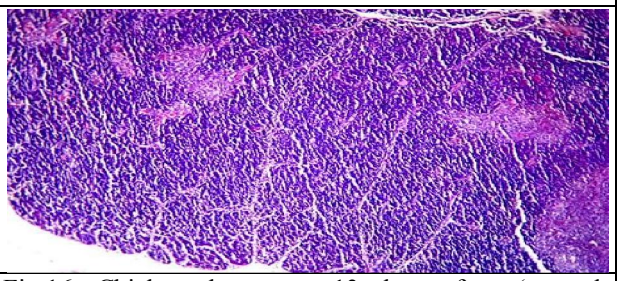


Fig.16: Chicken thymus at 13 days of age(control group) showing normal lobules H&E (X 100).

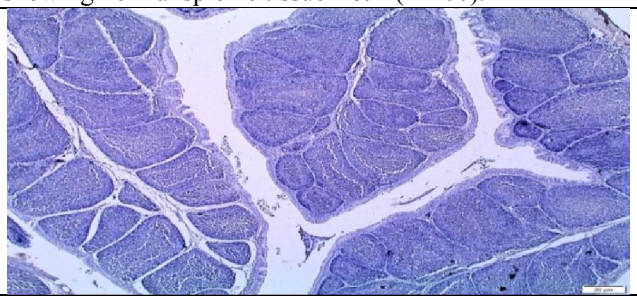


Fig.17: Control bursa from 27 day old saso chicken showing negative immune-peroxidase reaction against IBVD.
IIP counter stain with Mayer's hematoxylin X 100.

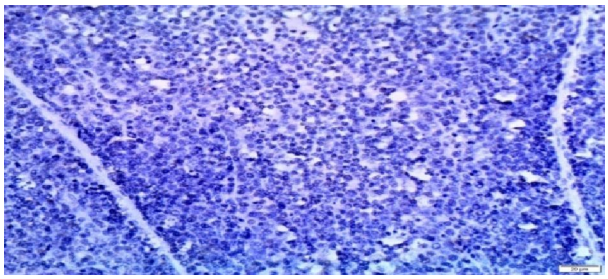


Fig.18: Control bursa of 41 day old saso chicken showing negative immune-peroxidase reaction against IBVD.
IIP counter stain withMayer'sHematoxylinX 400.

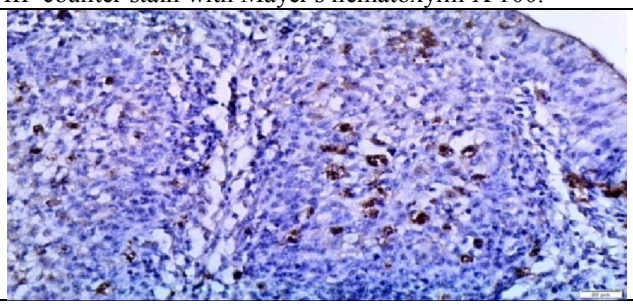


Fig. (19): BF 6 days post challenge at 7 days of agewith 228-E IBDV. the IBDV was detected as a course intra-cytoplsmic golden brown granules in the follicular lymphocytes.
IIP counter stain withMayer's Hematoxylin X 400.

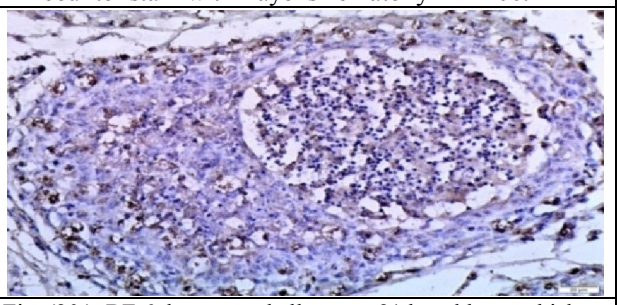


Fig. (20): BF 6 days post challenge at 21day old saso chicken infected with 228-E IBDV., IBDV was seen in some follicular lymphocytes and inter-follicular septa, as a fine and course intra-cytoplasmic golden-brown granules. Follicles also shows areas of complete lymphoid destruction IIP counter stain with Mayer's Hematoxylin X 200.

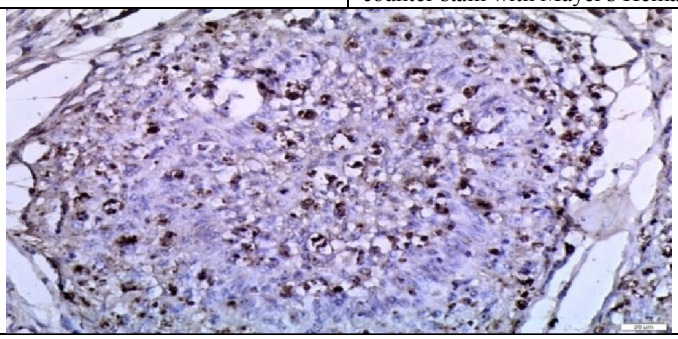


Fig. (21): chicks BF 6 days post challenge at 35 day old in chicken infected with 228-E IBDV. Showing IBDV in the cytoplasm of follicular lymphocytes IIP counter stain with Mayer'sHematoxylinX 400.

Table (2): MDA for IBD., beside morbidity, mortality of native saso chicks inoculated at 7,21 and 35 days of age using (IBDV. 228-E).

	Infected		Control		QAGPT for MDA (GMT ± S.E)
	Morbidity	Mortality	Morbidity	Mortality	
1	-	-	-	-	1.75±.24
7	0	0	0	0	0
21	(15/25) 60%	(3/25) 12%	0	0	-
35	(20/25) 80%	(2/25) 8%	0	0	-
This table was taken from the series of Bayoumie et al (2016).					

Table (3): Hematological assay 6 days post installation of (IBDV. 228-E) at each time mark., as described in table (1).(n=5)

AGE / parameter	At 13 day		At 27 day		At 41 day	
	Infected	Control	Infected	Control	Infected	Control
	Mean ±S.E	Mean ±S.E	Mean ±S.E	Mean ±S.E	Mean ±S.E	Mean ±S.E
HGB (g/dl)	6.18 ± 0.36	5.84 ± 0.29	6.78 ± 0.35	8.23 ± 0.31	9.34 ± 0.53	9.62 ± 0.15
RBCS (10^6 / ul)	1.93 ± 0.08	1.83 ± 0.04	1.92 ± 0.1	2.28 ± 0.08	2.57 ± 0.16	2.55 ± 0.03
HCT (%)	26 ± 0.93	24.76 ± 0.73	25.32 ± 1.33	29.1 ± 0.65	32.62 ± 1.96	33.36 ± 0.36
MCV (fl)	134.98 ± 2.14	135.34 ± 1.3	132.12 ± 1.6	128.05 ± 1.62	126.78 ± 0.55	130.98 ± 1.6
MCH (pg)	33.15 ± 0.96	31.88 ± 0.93	35.4 ± 0.48	36.18 ± 0.54	36.36 ± 0.73	37.72 ± 0.61
MCHC (g/dl)	24.55 ± 0.67	23.54 ± 0.45	26.8 ± 0.07	28.23 ± 0.56	28.66 ± 0.47	28.88 ± 0.15
WBCS (10^3 / ul)	1.52 ± 0.12	1.56 ± 0.08	2.44 ± 1.57	4.87 ± 2.05	n.d.	n.d.
HETRO (%)	15.1 ± 2.88	11.02 ± 1.13	59.8 ± 8.66	9 ± 1.03	47.56 ± 4.75	11.16 ± 3.27
LYMPH (%)	80.03 ± 2.45	83.72 ± 1.02	32.56 ± 6.72	77.88 ± 6.21	36.94 ± 8.12	78.86 ± 5.35
MONO (%)	4.6 ± 0.63	4.92 ± 0.51	6.38 ± 2.81	12.38 ± 5.56	15.5 ± 4.79	4.74 ± 1.64
EO (%)	0.28 ± 0.28	0.34 ± 0.22	1.26 ± 1.26	0.75 ± 0.45	0	5.14 ± 3.12
BASO (%)	0	0	0	0	0	0.1 ± 0.1
PLT (10^3/UL)	4.25 ± 0.95	5.2 ± 0.73	8 ± 3.11	7 ± 2.74	25.4 ± 6.38	5.2 ± 1.5
Bleeding Time	148.4±2.420	117.8±2.51	148.6±1.43	104.4±2.15	248.0±4.63*	101.2±1.39
Cloting Time	168.2±2.59*	63.4±1.80	192.0±2.6**	62.4±2.561	332.0±7.8**	125.25±2.05
n.d.= not done * P< 0.05 ** P< 0.01						

Table (4): B.B.I6 days post installation of (IBDV228-E) at each time mark.,as described in table (1).(n=5)

Age		Infected					Control		
		Body wt	bursal wt	B:B ratio	B:B index	Bursalatrophy	Body wt	Bursal wt	B:B ratio
13 day	Mean ±S.E	101.82±4.8	.32±.101	0.003	0.6639	+	104.76±2.5	.5±.03	0.0047
	Std	10.89	0.2280	0.002	0.4850		5.750	0.070	0.00052
	V	11.657	.052				33.063	.005	
27 day	Mean ±S.E	164.2±12.3	.5±.0001	0.003	0.657	+	186.6±3.24	.92±.03	0.0049
	Std	27.65	0	0.0006	0.136		7.266	0.083	0.00054
	V	7.700	0				52.800	.007	
41 day	Mean ±S.E	370.8±27.1	1.0±.242	0.0029	0.6148	+	503.8±26.6	3.28±.13	0.0066
	Std	60.64	0.5431	0.001	0.4015		59.675	0.311	0.0012
	V	36.200	.295				35.200	.097	
+ = indicate occurrence of bursal atrophy									

Table (5): Uric acid and creatinine in (mg/dl) 2,4 and 6 days post installation of (IBDV.228-E) at each time mark., as described in table (1).(n=5)

age		Uric acid (mg/dl)		Creatinine(mg/dl)	
		infected	Control	infected	Control
9 day	Mean± S.E	14.32±.2.3	9.28±1.35	.317±.02	.14±.017
	Std	4.63	2.7	.043	.035
	V	21.44	7.33	.002	.001
11 day	Mean±S.E	18.02±3.57	9.03±.38	.162±.018	.157±.021
	Std	7.14	.77	.037	.042
	V	50.98	.602	.001	.002
13 day	Mean±S.E	15.25±4.49	9.78±1.22	.235±.014*	.177±.014
	Std	8.98	2.44	.028	.029
	V	80.7	5.9	.001	.001
23 day	Mean±S.E	16.77±1.62	13.57±1.85	.277±.052	.255±.035
	Std	3.256	3.71	.105	.070
	V	10.6	13.7	.011	.005
25 day	Mean±S.E	19.7±2.63	12.8±1.79	.31±.031	.25±.03
	Std	5.26	3.59	.062	.070
	V	27.767	12.902	.004	.005
27 day	Mean±S.E	17.0±1.75	12.82±1.214	.287±.036	.25±.035
	Std	3.504	2.429	.0732	.07047
	V	12.28	5.903	.005	.005
37 day	Mean±S.E	19.42±3.22	11.35±1.226	.295±.0317	.25±.036
	Std	6.44	2.45	.063	.073
	V	41.542	6.017	.004	.005
39 day	Mean±S.E	13.60±2.59	11.10±.54	.375±.02	.275±.017
	Std	5.19	1.08	.040	.034
	V	26.98	1.167	.002	.001
41 day	Mean±S.E	22.9±4.11	11.6±.92	.41±.011	.33±.014
	Std	8.2	1.85	.023	.029
	V	67.707	3.433	.001	.001

* P< 0.05,

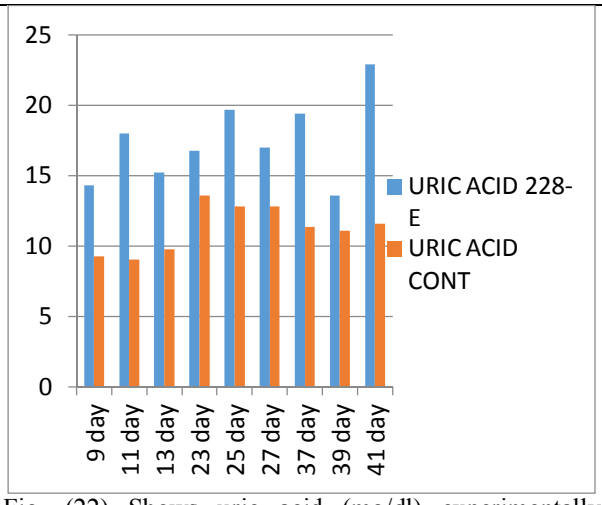


Fig. (22) Shows uric acid (mg/dl) experimentally inoculated saso chicks with (IBDV. 228-E) at different age intervals compared with control.

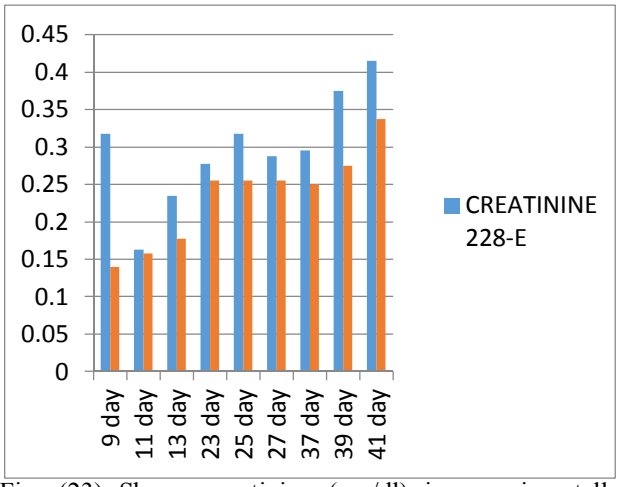
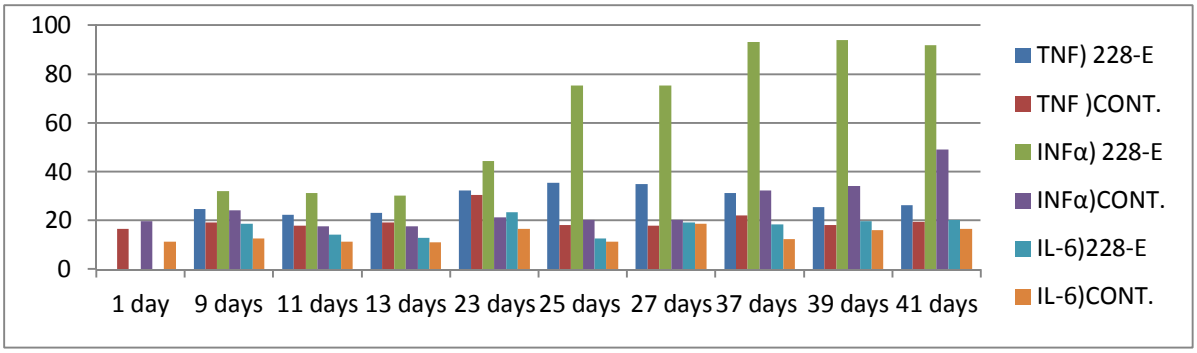


Fig. (23) Shows creatinine (mg/dl) in experimentally inoculated saso chicks with (IBDV. 228-E) at different age intervals compared with control.

Table (6): Elisa results of cytokine storm component(pg/ml) for ($INF_{\alpha 1/13}$, TNF, IL-6) at one day of age., then at 2,4 and 6 days post installation of (IBDV. 228-E) at each time mark as described in table (1).(n=5)

age		TNF		INF α 1/13		IL-6	
		infected	Control	infected	Control	infected	Control
1 day	Mean ±S.E		16.51 ±.903		19.68 ± 1.39		11.20 ±.375
	Std		1.80		2.79		.751
	V		3.267		7.834		.564
9 days	Mean ±S.E	24.70 ±1.79	19.12 ±1.3	31.97 ± 4.0	24.26±.78	18.61 ± 1.5	12.56 ±.237
	Std	3.59	.264	8.17	1.56	3.17	.474
	V	12.925	.070	66.794	2.461	10.078	.225
11 days	Mean ±S.E	22.42 ± 1.78	17.78 ±.204	31.23 ± 1.4	17.52 ±.645	14.24 ±.6	11.23 ±.33
	Std	3.57	.408	2.86	1.29	1.323	.677
	V	12.753	.167	8.198	1.667	1.750	.458
13 days	Mean ±S.E	23.16 ± 1.92	19.14 ± 1.33	30.24 ± 2.4	17.66 ±.462	12.93 ±.53	11.08 ±.142
	Std	3.84	2.66	4.99	.92	1.07	.284
	V	14.812	7.117	24.953	.854	1.157	.081
23 days	Mean ±S.E	32.39 ± 2.48	30.41± 1.249	44.40 ± 5.4	21.23 ±.2500	23.30 ±.5	16.66 ±.426
	Std	4.97	3.82	10.802	.50	1.11	.85
	V	24.714	14.629	116.700	.250	1.240	.728
25 days	Mean ±S.E	35.41 ± 1.24	18.10 ± 2.95	75.2 ± 3.8	20.20 ±.41	12.68 ±.93	11.36 ±.673
	Std	2.49	5.91	7.6	.84	1.87	1.35
	V	6.241	35.0	59.287	.693	3.476	1.814
27 days	Mean ±S.E	34.9 ± 2.39	17.77 ± 1.18	75.21 ± 3.8	20.21 ±.4162	19.23 ± 1.5	18.72 ±.853
	Std	4.78	2.37	7.69	.83	3.14	1.7
	V	22.87	5.633	59.287	.693	9.89	2.912
37 days	Mean ±S.E	31.18 ± 2.77	22.09 ±.0	93.17 ± 3.5	32.34± 4.78	18.4 ±.76	12.3 ±.0
	Std	2.37338	0	71.27	9.57	1.5	0
	V	30.574	0	508.6	91.667	2.337	0
39 days	Mean ±S.E	25.37± 1.9	18.14 ±0	93.9 ± 1.5	34.03 ± 4.787	19.8 ±.57	16.1 ± 0
	Std	3.8	0	21.08	9.57	1.159	0
	V	14.504	0	444.727	91.667	1.345	0
41 days	Mean ±S.E	26.16 ±.81	19.35 ±.657	91.90 ± 3.0	49.10 ± 1.568	20.08 ±.97	16.5 ±.57
	Std	1.6	1.3	6.01	3.1	1.9	1.2
	V	2.662	1.729	36.19	9.8	3.8	1.3



(Fig. -24): to simplify table (6).

Table(7): Mean pathological score of bursae6 days post installation of (IBDV228-E) at each time mark as described in table (1).(n=5)

Age	Infected			Control		
	Individual lesion score	Mean score	BBI	Individual lesion score	Mean score	BBI
13 days	(2/2/2/2/3)	2.2	0.6639*	(1/1/1/1/1)	1	-
27 days	(5/5/5/5/5)	5	0.657*	(1/1/1/1/1)	1	-
41 days	(5/4/4/3/3)	3.8	0.614*	(1/1/1/1/1)	1	-
*- positive immunosuppression						

4. Discussion

Several studies on the pathogenesis of IBDV infections have been conducted, **Skeeles et al. (1979$_{a,b}$)** attempted to demonstrate that the hemorrhagic lesions were a consequence of formation of immune complexes, as proposed by **Ivanyi and Morris (1976).** Histologic lesions in the cloacal bursa resemble an Arthus reaction (necrosis, hemorrhage, and large numbers of polymorphnuclear leukocytes) this reaction is a type of limited immunologic harm brought on by antigen-antibody-complement complexes that induce chemotactic factors, which cause hemorrhage and leukocyte infiltration. They found that 2-week-old chickens 72 hours PI had little complement compared with 8-week-old chickens. They proposed that the reason why 2-week-old chickens did not develop Arthus-type lesions was a lack of sufficient complement.

Ingrao et al. (2013) said the expression of (Heraclitus) *{There is nothing lasting., with the exception of change}*. Indeed., if we look to pathogenic interpretation of IBDV early and nowadays. Recent interpretation by **Van den berg (2008)**clarified the pathogenesis of IBD as follow., the target organ of IBDV is the bursa of Fabricius at its maximum development, where B lymphocytes matures in avian species**, Bayoumie et al. (2009)** specified that IBDV is dependent upon bursal ontogeny and maturation. The severity of the disease is directly relatedto the number of susceptible cells present in the bursa of Fabricius. This was apparent in the present study when we look to the recorded mortality and morbidity when (IBDV.228-E) was given at 7,21 and 35 days of age as seen in **(table-2).** The recorded symptoms and post mortem (PM) lesions as seen in **(figs. 1-5)** resembles symptoms and PM lesion known for IBD **Eterradossi and saif (2013).**

Ingrao et al. (2013) Defined pathogenesis as the method used by IBDV to cause injury to the host with mortality, disease and/or immunosuppression. Indeed, vvIBDVs produce disease signs similar to classical type 1 infections but the acute phase is exacerbated and more generalized in the affected flock. The incubation period is very short, 2–3 days. In acute cases, birds are exhausted, prostrated, dehydrated, suffers from aqueous diarrhea, and their feathers are ruffled. Mortality commences on the third day of infection, reaches a peak, then drops rapidly, and the surviving chickens recover a state of apparent health after 5–7 days.

Van den berg (2008) further interpreted that, during the acute phase of IBD, there is a dramatic infiltration of T cells around the site of virus replication, including the bursa of Fabricius., this was quiet evident in **(Figs.9,10 and 11)** in the present study. T lymphocytes don't bolster viral replication but are activated and display up regulation of cytokine genes that affects macrophage work with an exacerbated production of pro-mediators such as Interferon (IFN-1), Tumour necrosis factor (TNF- α), Interleukin (IL-6) or (IL-8). This cytokine storm induces a shock in the bird, which becomes prostrated and reluctant to move. This was seen in the present study **(Fig -1)**.

Van den berg (2008) stated that the severity of mortality could be linked to the magnitude of the provoked cytokines. Here several question are forced., {a- what is the reason for the variation in IBDV pathogenicity ?., b- what is the role of age in IBDV pathogenicity ?., c-what is the estimate of breed susceptibility in the total expressed IBD picture ?.

Ruby et al. (2006) demonstrated significant interline differences in the regulation of cytokine genes of the inflammatory response, which were up-regulated only in the resistant compared to the susceptible chicken lines. **Tippenhauera et al.,(2013)** found that host genotype influences IBD pathogenesis in chickens by modulation of T cells responses and cytokine gene expression. This could interpret our early observation with challenged broiler breed **Bayoumie (1997)**., and in the present study when we used the native saso breed{(native saso breed is a crossbred type of red shaver male and native chicken female, it is also a double purpose breed (Meat producing and egg producing)} the native saso breed is highly sensitive for IBDV compared to broilers, these findings fortifies the earlier work of**Bayoumie et al. (2009).** Our recorded observation could be deciphered by the work of **Tippenhauera et al. (2013)**since they affirmed that genetic differences exist in the cytokine response after virus infection in chicken. The type I IFN (IFN-a) and IFN-c mRNA expression was up-regulated in spleen and BF of SPF (Wh-LT) and (Ross-BT) chickens, which partially coincided with the detection of these cytokines in serum of infected birds. In (Cobb-BT), (Br-LT) and (Wh-LT) chickens, the IFN-a gene expression in spleen was down-regulated but up-regulated in the BF. The release of the bioactive cytokines was delayed in these genetic backgrounds compared to the other groups. We may speculate that this delay may allow better regulation of the T cell response and reduce the effect of cytokine storm as mentioned by **Perruche et al., (2009); Alberts et al., (2010)**.

Lunney (1998); Kim et al. (2000); Sharma et al. (2000); Rautenschlein et al. (2002); Beal et al. (2004); Khatri et al. (2005); Khatri and Sharma (2006); Rautenschleinaet al. (2007$_{a,b}$); Xianghai et al. (2007); Gao et al. (2008); Khatri and Sharma (2008); Haiwen Liu et al. (2010); Aricibasi et al.,(2010); Dhinakar et al.,(2011); Carballed et al., (2011); Rauf et al. (2011$_a$); Rauf et al. (2011$_b$); Jain et al (2013); Tippenhauera et al.,(2013); Ingrao et

al. (2013); Carballed et al., (2014); Sanying Wang et al. (2014); Smith et al. (2015); Hui et al. (2015) were investigating the cytokine storm gene expression. But in the present study we had observed that it well be more fitting to quantify the provoked cytokine storm component in particular (IL-6, TNF, INFα $_{1/13}$) on an ELISA scale at three age focuses (2^{nd}, 4^{th} and 6^{th} day following each administration of (IBDV.228-E) at (7, 21 and 35 days of age). Second issue, is., we did intentionally utilized the hot vaccinal strain of IBDV termed 228-E to open the entry way for other workers to proceed advancement studies in this line of work. Third issue is that., ELISA kits used were intended for human examination for their feasibility. Personal communication with the producing company informed us that {as TNF, we did not found its protein sequence on the net, so we are not sure whether E0133h can test in chicken., andforIL-6 the homology of human and chicken protein sequence is 32.787% (molecular synapse)., but for Interferon $\alpha_{1/13}$, human and chicken protein sequence homology is 18.687%(molecular synapse). Recorded results in the present study uncovered that the mean pg/ml of TNF at three age marks following (IBDV.228-E) administration at 7 days of age was 23.4 while it was 18.6 in the control, the mean pg/ml of INFα $_{1/13}$ at three age marks following (IBDV.228-E)administration was 31.1 while it was 19.8 in the control on the other hand the mean pg/ml ofIL-6 at three age marks following (IBDV.228-E)administration was 15.2 while it was11.6 in the control this picture had changed when (IBDV.228-E)was administrated at 21 days of age since the mean pg/ml of TNF was 34.2 following (IBDV.228-E)administration while it was 22 in the control., and the mean pg/ml of INFα $_{1/13}$ was 64.9 following (IBDV.228-E)administration while it was 20.5 in the control., as for IL-6 the mean pg/ml of IL-6 was 18.4 following (IBDV.228-E)administration while it was 15.5 in the control. This picture had changed again when (IBDV.228-E)was administrated at 35 days of age since the mean pg/ml of TNF was 27.5 following (IBDV.228-E)administration while it was 19.8 in the control., the mean pg/ml of INFα $_{1/13}$ was 92.9 following (IBDV.228-E)administration while it was 38.4 in the control., and the mean pg/mlof IL-6 was 19.4 following (IBDV.228-E)administration while it was 14.9 in the control**(table 1,2 and 6 Fig-24)**. **Ingrao et al. (2013)** specified that there is developing proof for the role of innate immunity, particularlypro-inflammatory mediators, in the pathogenesis of IBD. Indeed, during the acute phase of IBD and as early as 1 day post-infection, there is a dramatic infiltration of CD4 cells, CD8+cells and macrophages at and close to the site of virus replication, mainly in the BFas mentioned by **Sharma et al. (2000); Withers et al. (2005).** BursalT cells are activated and exhibit up-regulation of gene transcription of pro-inflammatory cytokines e.g. ChIL-1b, ChIL-6, CXCi2 andC hIFN-c **Eldaghayes et al. (2006**). Abnormalamounts of systemic ChIFNc and ChIL-6 were additionally seen during the acute phase following vvIBDV challenge demonstrating the role of an exacerbated innate immune response in the acute phase of the disease, leading to a socalled‘ ‘cytokine storm’’ **Rauw et al., (2007)**. The ChIFN-c up-regulationwas correlated with production of IL-12_a, an increased level of IL-18 mRNA in splenic macrophages and pro-inflammatory factors including ChIL-1_b, ChIL-6, and inducible nitric oxide synthetase (iNOS)**.,** this may advance cellulardys-regulation and accentuate tissue destruction **Kim et al., (1998).** Additionally, macrophages and monocytes enacted by IBDV are directly activated producing high levels of mediators such as pro-inflammatory cytokines, interleukin-1 (IL-1) and (IL-6), chemokines (IL-8 and MIPa and nitric oxide)**Kim et al., (1998); Khatriet al. (2005); Rauf et al.,(2011$_b$)** The signal transduction pathways involved in macrophage activation have also been inspected by **Khatri and Sharma (2006).** The varied morbidity and mortality recorded in the present study as seen in (table -2) where we can find low morbidity plus high mortality when birds were given (IBDV.228-E) at 21 day of age could be ascribed to the synergistic impact between the released INFα $_{1/13}$ and TNF. At 35 days of age INFα $_{1/13}$ increased solely to a very high levels so morbidity was high and mortality was low., then morbid birds survived., this observation could be interpreted because synergistic impact between INFα $_{1/13}$ and TNF was lost (table-6, Fig.- 26). With respect to the consequences of administration of (IBDV.228-E) at 7 days of age the virus had replicated in the B lymphocytes causing edema and necrosis in the center of the bursa lymphoid follicles (Fig. -6) this impact unquestionably will be manifested with immunosuppression confirmed by the results of BBI (table -4)., it also worth to specify thatno morbidity or mortality were recorded at this point., these findings were synchronized with lowprovoked cytokines at this age point., we think it might be the result ofcredulous nature of immune system at this early age.

One of the conflicting issues is the distinction in pathogen city between IBDV isolate. **Eterradossi and saif (2013)**specified that IBDV possessfive viral proteins assigned VP1, VP2, VP3, VP4, and VP5 are perceived with estimated molecular weights of 97 kD, 41 kD, 32 kD, 28 kD, and 21 kD, respectively. And they also possess additional protein termedVPX. The VP2, VP3, and VP1 are the structural proteins of IBDV. In serotype 1 viruses, they constitute 51%, 40%, and 3% of the virus proteins, respectively. **Van den berg (2008)** stated th**at** there is a growing

evidence for a role of pro-inflammatory cytokines in the pathogenesis of IBD. Indeed, during the acute phase of IBD, there is a dramatic infiltration of T cells around the site of virus replication, including the bursa of Fabricius, spleen and caecal tonsils. T lymphocytes do not support viral replication but are activated and exhibit upregulation of cytokine genes that has an effect on macrophage function with an exacerbated production of pro-mediators such as interferon (IFN)1, tumour necrosis factor (TNF) α, interleukin (IL) 6 or IL8. This cytokine storm induces a shock in the bird, which becomes prostrated and reluctant to move. In light of these two ideas and the outcomes acquired in the present study we can presume that the distinction in IBDV pathogenicity is brought about by contrast in VP substance of the isolate and the ability of the virus to replicate faster., thus autonomously replicating IBDV will generate a bigger foreign antigenic mass of the viral protein evoking severe shock reaction in chicken., this can interpret varied symptoms and mortality met with IBDV.

With respect to the third conflicting issue., the age and its relation to the total IBD picture at the three age points. In the present study and the earlier work of **Bayoumie et al. (2009)**., it seem that distinction in disease picture will be associated to the bursal maturity and the maturation of the immune system.

In the present study the lymphoid follicles of bursae from negative control chicks at 13, 27 and 41 day of age showed normal lymphoid intensity in both cortical and medullary zone in both histopathological sections and immunohistochemistry examinations **Fig (18)**. Infected bursae 6 daysafter |(228-E)administration at 7 days of age showed a mild edema with necrotic debris in the center of lymphoid follicles beside scattered heterophiles and lymphoid depletion., its mean score was (2.2) **(table-7) (Fig.6)**., the necrotic changes seen were represented by karyorrehexis. Hyperemic blood vessels and a few extravasated erythrocytes were also seen., the depleted lymphoid follicles showed proliferation of the reticuloendothelial cells and mild fibroblastic proliferation in interfolliculartissue. Infectedbursae 6 days after |(228-E) administration at 21 days of age., the necrotic changes were more prevalent and was accompanied by minute cystic spaces in both cortex and medulla in the majority of the lymphoid follicles **(Fig. 8 and 9)** lymphoid depletion was (scored as 5) **(table-7)**. Moreover extensive edemas were seen in the majority of lymphoid follicles beside inter follicular spaces and hyperplasic covering epithelium **(Fig.9)**. Infected bursae 6 days after |(228-E) administration at 35 days of age showed few heterophilic infiltrations together with prominent fibroplasias in the interfollicular tissues., proliferation of reticuloendothelial cells in both cortex and medulla of depleted lymphoid follicles was seen. Edema and necrotic changes in all depleted and atrophied lymphoid follicles was accompanied by extensive fibroplasias in interfollicular tissue **(Fig.10)** lymphoid depletion was score as (3.8)**(table -7)**., beside Hyperplasia of the covering epithelium and proliferation of reticuloendothelial cells in both cortex and medulla. Lesions induced in spleen of infected chicks with **(IBDV 228-E)** at the three age points mentioned in **(table -1)** is represented by Hyperplastic reticuloendothelial cells in all splenic lymphoid follicles beside dilated splenic sinusoids, and depletion of lymphoid follicles and splenic sinuses., beside severe congestion, hemorrhages and hemosiderosis extensive necrosis of the spleen, normal spleen of control birds is seen in **(Fig.15)**. Thymus of infected chicks with (IBDV. 228-E) at the three age points mentioned in **(table -1)** the recorded lesion was extensive hemorrhage, hyperemic blood vessels and edema in all regions of thymic lobules beside necrotic and minute cystic spaces in cortex and medulla**.** A few heterophilic proliferations and hyperplasic reticuloendothelial cells could be seen within the medulla of a few thymic lobule (**Fig.7, 12**). Normal thymus of control birds is seen in **(Fig.16).** These pathological alteration resembles that recorded for VVIBDV field isolates of gumboro according to **Eterradossi and saif (2013).**

Immunohistochemical examination was made 6 days following the challenge at the age of 7, 21 and 35 days of age in whichbursa showed negative reaction against IBVD (Fig.17, 18)whilethe virus was seen in some follicular lymphocytes and inter-follicular septa, as a fine and course intra-cytoplasmic golden-brown granules (Fig.19,20 and 21)was very much helpful to confirm viral replication in the examined bursae **Eterradossi and saif (2013)**.

Results of previous work of**Bayoumie et al., (2009)** had led us to rehash the original copy precisely with just two contrasts 1st issue we utilized the hot strain of IBDV vaccine termed 228-E trusting somebody may proceed with our arrangement., so the 228-E will be effortlessly gotten. 2nd issue is we included several research criteria that were not included in the earlier work for example (immunohistochemistry, cytokine storm evaluation for IL-6, IFN and TNF) to have a superior view in understanding the patho-biology of IBD. Obtained results of complete blood picture, bleeding time, clotting time and kidney functions beside the pathological lesion score in the bursa following (IBDV.228-E) instillation at 7,21 and 35 days of age in native saso chicks as seen in **(table. 3-5and 7, beside descriptive Fig 22,23 and 24)**were comparable to the previous results recorded in**Bayoumie et al.,**

(2009) although the utilized viral isolate was not similar.

Other issue, we think that it needs further investigation., which is thepurported (nephrogenic affinity of IBDV**). Eterradossi and Saif (2008)** stated that PM examination of birds that died during the acute phase of vvIBD, the BF is the principal diagnostic organ: it is turgid, oedematous and sometimes haemorrhagic and turns atrophic within 7–10 days. In addition, dehydration and nephrosis with swollen kidneys are common and ecchymotic hemorrhages in the muscle and the mucosa of the proventriculus are observed in many affected birds. **Hirai and Calnek, (1979); Hirai et al., (1981); Rodenberger et al., (1994)** mentioned that IBDV replicates in theimmunoglobulin M-bearing B lymphocytes. **Van den berg (2008)** mentioned that IBDV., instigate a cytokine tempest that shocks the affected bird. In the present study as seen in**(Fig-1,3)** we believe that shock, inability of the shocked bird to reach water beside watery diarrhea causes dehydration which may be the cause behind the observed kidney lesion.

Conclusion

This work is a trial to advance researchof some patho-biological aspects with IBDV added to our series in this regard. Now we are able to mention that IBDV is not a deadly infection since it causes no disease when inoculated at 7,14 or 70 days of age., disease problems are only seen in the period from 3-6 week of age., it became obvious for us that the IBD is an anaphylactic entity rather than inflammatory one., after studying the provoked cytokine components namely (IL-6, INFα 1/13 and TNF) at three age points for each age of (IBDV.228-E)administration, we were additionallyready to decide the synergistic impact between the analyzed cytokine in the IBDV pathogenesis which could help the elucidation of mortality and morbidity seen in (table -2).

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