Comparative Protective Efficacy of Combined Vaccines against Newcastle Disease and Infectious Bronchitis Viruses in Broiler Chickens in Egypt

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ABSTRACT

A study has been carried out to evaluate different local and imported combined inactivated NDV+IBV vaccines and investigate its ability to protect experimental broiler chickens against challenge with wild ND and IB viruses. Two hundred and eighty commercial one-day-old broiler saso chicks were divided into 8 groups (35 birds each). Groups 2, 3, 4&5 were vaccinated at one-day-old with live hitchner + IB Primer. Then all groups at 7 days old were boosted with inactivated combined NDV+IBV vaccines with different adjuvants except for group 1 & 2. Group 1 was kept as unvaccinated control. At 28 days all groups were challenged with a dose of 105.5 EID50/bird of Variant 2 genotype (1494) IBV through oculo-nasal rout. Blood samples were collected randomly from 10 birds from each group at day 7, 14, 21, 28, 35 and 42 . Organs of 3 birds/each group were collected (trachea, bronchi and kidneys) for PCR at 3, 7 and 10 days post vaccination and post challenge as well. Groups 3 &4 showed high protection with all serological tests. Based on our findings Vaccine Manufactured with the Local Strains & Montanide ISA 71 proved to be a good Vaccine as the groups received it as booster after priming with live vaccination, showed high and good protection against IBV especially after challenge with wild live IBV virus where the virus couldn't be detected in trachea & kidney after 7 days post challenge even by using the sensitive rRT-PCR.

Keywords: infectious bronchitis disease, Newcastle disease, Broilers, inactivated combined NDV+IBV vaccines, MONTANIDE ISA 70VG adjuvant, MONTANIDE ISA 71 adjuvant, Protection.

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INTRODUCTION

Newcastle Disease (ND) and infectious bronchitis (IB) are important diseases in the poultry industry and cause great losses (King & Cavanagh, 1991; Tu et al., 1998). Infectious bronchitis virus (IBV) is prevalent in all countries with an intensive poultry industry, with the incidence of infection approaching 50% in most locations. Vaccination is only partially successful due to the continual emergence of antigenic variants. Infectious bronchitis has a significant economic impact; in broilers, production losses are due to poor weight gains, condemnation at processing and mortality, whilst in laying birds, losses are due to suboptimal egg production anddown grading of eggs. The majority of IBV strains cause tracheal lesions and respiratory disease with low mortality due to secondary bacterial infections, primarily in broilers. Nephropathogenic strains, in addition to tracheal lesions, also induce prominent kidney lesions with mortality of up to 25% in broilers. Strains of both pathotypes infect adult birds and affect egg production and egg quality to a variable degree (J. Ignjatovic and S. Sapats, 2000). Also, Newcastle disease (ND) is one of the most infectious, highly contagious, fatal viral diseases of chickens, characterized by respiratory, digestive, and nervous symptoms (Mishra et al., 2000). Control involves the use of biosecurity

procedures and vaccination (Mayahi et al., 2013), which is routinely used throughout the intensive poultry industry. The following factors are a feature of IB vaccination:a) immunity after vaccination is not long-lasting and re-vaccination is necessary, b) the selection of an appropriate antigenic type of the region is important, given the existence of wide antigenic variation, c) timing and method of vaccine application will vary for different flocks and may require adjustment according to practical experiences (J. Ignjatovic and S. Sapats, 2000). In order to reduce costs, vaccination using two or three vaccines simultaneously became a common practice in poultry production, such as a combined vaccine against ND virus (NDV) and IBV. Since the risk that, if it is present in excess, the Infectious Bronchitis vaccine may interfere with the response to the Newcastle Disease vaccine, the use of a combined product is preferable to the use of two separate vaccines given together (Cook, 2008).The objective of this study was to pursue comparative evaluation of different local and imported combined inactivated NDV+IBV vaccines and investigate its ability to protect experimental broiler chickens against challenge with wild ND and IB viruses.

MATERIALS AND METHODS

Birds, and Chicken embryos and chicks:

Specific pathogen free (SPF) chicks, and embryonated chicken eggs (ECEs) (Specific Pathogen Free Farm,KoumOsheim) were used for propagation and titration of live IBV strains and vaccines.Two hundred and eighty commercial one-day-old broiler Saso chicks were floor reared under strict hygienic condition in isolated experimental rooms (5X5/35). Birds were fed commercial ration Containing antimicrobial agents and growth promoters.

Vaccines:

A commercial combined inactivated trivalent virus vaccine of IB/ND (MEVAC IB+ND, 500 Ml , 0.5 ml/dose, MEVAC, Egypt) was used. The vaccine is formulated using 107 EID50/dose of the local isolates Eg/11539F (IB Classical) and Eg/1212B (IB Variant type 2), and 108 EID50/dose of NDV/Chicken/Egypt/11478AF/2011. The vaccine formula contained Montanide ISA 70VG (Seppic, France) as an adjuvant.

A commercial imported inactivated combined vaccine for chickens against IB, Newcastle Disease and Egg Drop Syndrome (Nobilis® IB multi+ND+EDS, 500 Ml,0.5 ml/dose , Intervet International B.V. BOXMEER – HOLLAND) was also used. The vaccine is formulated using ≥ 5.5 log2 VN Units/dose of serotype Massachusetts M41 (IB Classical) and ≥ 4 log2VN Units/dose D207/ strain D274 and related strains (IB Variant type 2) and ≥ 50 PD50 Units/dose of NDV (according to potency test). Commercial vaccines were administered according to the manufacturers' instructions. An experimental vaccine was formulated as MEVAC IB+ND with substitution of Montanide ISA 70VG (Seppic, France) with Montanide ISA 71 (Seppic, France).

Experimental design:

To test the effect of adjuvant replacement on vaccine efficacy in broilers, the experiment was conducted on birds from 0-42 days old. At the beginning of the experiment, one-day old birds were divided into 8 groups (35

birds each) treated as described in Table (1).

Table 1: Experimental Design

Groups	Vaccine		Priming With Live vaccine	Boosting with Inactivate vaccine	Challenge	Sample Type & Time
	Live	Inactivated				
1	NA	NA	Unvaccinated	Unvaccinated	At 28 days old	Blood samples atday 7, 14, 21, 28, 35, 42 for sera separation (trachea, bronchi and kidney)at 3,7,10 days post vaccination& at post Challenge for antibody titer monitoring by HI and ELISA
2	Hitchner + IB Primer	-	One – day old	-		
3	Hitchner + IB Primer	Commercial MEVAC IB+ND Montanide ISA 70VG		At 7 days old		
4	Hitchner + IB Primer	Experimental MEVAC IB+ND Montanide ISA 71				
5	Hitchner + IB Primer	Commercial Nobilis® IB multi +ND+EDS				
6	-	Commercial MEVAC IB+ND Montanide ISA 70VG	-			
7	-	Experimental MEVAC IB+ ND Montanide ISA 71				
8	-	Commercial Nobilis® IB multi +ND+EDS				

NA: Unavailable

IB Primer: live attenuated vaccine contains strains H120+D274 of IBV.

Sample types and sampling schedule:

Blood samples were collected from 10 birds selected randomly from each group at day 7, 14, 21, 28, 35 and 42 for sera separation.Organs of 3 birds/each group were collected (trachea, bronchi and kidneys) for PCR at 3,7 and 10 days post vaccination and post challenge as well.

Challenge:

Ten birds from each group were isolated and challenged at 28 days old with a dose of 105.5 EID50/bird of Variant 2 genotype (1494) IBV through oculo-nasal rout. The challenge virus has been obtained from Dr. Magdy El Kady, Dean of the Faculty of Veterinary Medicine, Bani-Suef University. The birds were kept under observation for 14 days post challenge for the development of clinical signs of the disease (coughing, nasal discharge and rales) and P.M. lesions or mortality.

Hemagglutination-Inhibition (HI) test: (Alexander et al., 1983)

Collected Sera were tested for determining antibody titers for NDV and IBV by HI test based on OIE recommendations using both M41 and D274 antigens. Titers are expressed as log2 of the reciprocal value of the highest serum dilution showing complete HI.

Enzyme immune assay: (OIE, 2013)

The enzyme-linked immunosorbent assay (ELISA) was used to measure antibody levels to Avian infectious bronchitis virus (IBV).Serum samples were assayed in single dilutions using a commercial total antibody ELISA

(Biochek, Netherland) according to the manufacturer's Instructions.

Virus isolation:

- Preparation of samples for viral isolation and RNA extraction: (Jose et al., 2000)

Trachea and Kidney samples were collected and then frozen at below-10°C. After thawing, the tissue homogenates (10% W/V) were prepared in sterile saline 0.85% containing 1000 IU/mL penicillin, 1.0 mg/ml streptomycin. By disrupting tissue using sterile mortar and pestle, the homogenates were then centrifuged at 3000 rpm for 10 min, and the supernatant was further passed through 22 µm membrane filter. Sterility of the inocula was checked pre-inoculation by culturing on nutrient agar and sabouraud's glucose agar. These material was examined for presence of IBV by passage in embryonated eggs. Also these material was examined for presence of viral RNA other than IBV as AIV-H9, AIV-H5, VNDV or IBDV.

- Specific Pathogen Free (SPF) embryonated chicken egg inoculation (Gelb and Jackwood, 1998):

Kidney or trachea samples were taken from chickens and homogenized (1:10 W:V) in sterile saline contain antibiotics. The homogenate was then centrifuged and the supernatant was passed through 22µm filter then inoculated in embryonated eggs. For each sample to be examined, five 9-days-old embryonated specific pathogen free (SPF) eggs were used. The eggs were inoculated into the allantoic cavity with 0.2 ml of the samples, then incubated for 6 days and candled each day so that dead embryos could be recorded and removed. After 6 days of incubation, the eggs were chilled at 4°C. The allantoic fluid was collected and tested for haem-agglutination (HA) reaction with 10% chicken red blood cells (RBCs) solution (to exclude heam-agglutinating agents). Uninoculated SPF eggs were always included as control negative. Each sample was re-inoculated for four to seven passages to examine the embryonic lesion of IBV (curling and dwarfing).

- Rapid slide Haemagglutination (HA) test :

The test was carried out as outlined by Anon (1980). This was particularly useful in the rapid detection of HA activity in embryonic fluid using a 10% suspension of washed chicken RBC in saline (Beard, 1989).

Virus Titration:

Infected allantoic fluid of IBV isolates that have CAM (collected from inoculated eggs) homogenates found to be positive in AGP test against positive precipitating IB antisera, was diluted in sterile PBS by serially tenfold dilution [mixing 0.5 ml of infectious allantoic fluid with 4.5 ml of sterile PBS plus antibiotic (1000 IU/ml of penicillin and 1.0 mg/ml streptomycin) making 10 fold dilution] 10-1 through 10-10. The 10-1 through 10-10 dilutions were inoculated in 9-11day old SPF embryonated chicken eggs, 5 eggs were inoculated for each dilution (50 eggs total). Then 0.1 ml of the appropriate dilution was inoculated via allantoic sac route per embryo. The eggs were incubated for 7 days, candling daily. Deaths occurred during first 24 hours not used in calculation, as considered non specific deaths. On the seventh day post inoculation, all the survival eggs chilled in the refrigerator overnight and the embryos were examined for stunting, curling, clubbed down and kidney urates. Embryos exhibit one or more lesion were considered positive. Log 10

EID50 titer was determined using the method of Reed and Muench (1938).

Real-time RT-PCR (rRT-PCR)

Trachea and kidneys were collected for virus detection by rRT-PCR using quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA), with specific primers (Forwared: GCT TTT GAG CCT AGC GTT, Reverse: GCC ATG TTG TCA CTG TCT ATT G) and probe named (5' CAC CAC CAG AAC CTG TCA CCT C3') according to Callison et al., 2006.

Statistical Analysis

To evaluate the significance of some results obtained in the present study, the one-tailed Fisher's exact test was employed. One-way analysis of variance has been adopted using SAS software general liner models procedure (SAS Institute,1999). Mean values were assessed for significance using Duncan's multiple range test at$P< 0.05$.

RESULTS

Antibody response to NDV and IBV in broiler chickens:

The antibody response of the chickens to different vaccination programs using M41& D 274 antigen is shown in table 2. There has been a significant difference between control and vaccinated groups. The mean HI antibody titer to IBV started to increase after 7 days in groups 2,3,4 and 5 which received live vaccination at one day old. At 14 days (7 days after boosting with inactivated vaccine) there was an increase in HI titer in all groups (3-8), except for control no. 1 and group no. 2, but with significant increase in group no. 7 which received experimental MEVAC IB+ND ISA71alone and group no. 4 which received live priming with Hitchner-IB Primer vaccine followed by vaccination with experimental MEVAC IB+ND ISA70. The highest titer was noticed in group no. 3 at 28 days with a titer of 9.5 log2. Then the titers started to decrease at 42 days to range between 5 log2 and 8 log2.

The antibody response of the chickens to NDV different vaccination programs is shown in table 3. Groups 3 and 7 at 21 days were giving the highest titers 4.9 and 4.6 log2, respectively. These Groups continued to give a high titer in 28, 35 and 42 days old.

Table 2. Mean IB HI titer in broiler chickens vaccinated with different vaccination programs#.

Groups	Mean HI antibody Titer against IBV											
	7 days		14 days		21 days		28 days		35 days		42 days	
	M41	D274	M41	D274	M41	D274	M41	D274	M41	D274	M41	D274
1	2.4 ± 0.36	2.5*± 0.31	1.8 ± 0.47	1.1± 0.13	2.3 ± 0.77	1.5± 0.08	1.1 ± 0.32	0.5±0.0	0.3 ±0.1	0.5±0.09	0.3 ± 0.1	0
2	3.4 ± 0.20*	3.2+ 0.29	2.6 ± 1.6*	2.6± 0.43*	4.2 ± 0.11*	4.5± 0.92*	3.1 ± 0.13*	3.9±0.16 *	1.5 ± 0.2*	2.8±0.58*	1.3 ± 0.2*	2.2±0.41
3	3.4 ± 0.20*	3.2+ 0.29	4.3 ± 0.51*^{a}	5.1± 0.91*^{c}	8.2 ± 0.29*^{a}	8.7±0.69*^{a}	9.5 ± 0.71*^{a}	8.0±0.99 b	9.4 ± 0.71*^{a}	7.4±0.19^{c}	9.0 ± 0.33*^{a}	5.9±0.29^{c}
4	3.4 ± 0.20*	3.2+ 0.29	4 .0 ± 0.43*^{b}	5.8± 0.45*^{a}	7.6 ± 0.81*^{b}	8.5±0.88^{b}	8.6 ± 0.59*^{b}	8.4±0.54 *^{a}	9.4 ± 0.63*^{a}	8.2±0.71^{a}	9.4 ± 0.53*^{a}	6.5±0.71^{b}
5	3.4 ± 0.20*	3.2+ 0.29	3.9 ± 0.24*^{c}	5.6± 0.71*^{b}	6.4 ± 0.19*^{c}	5.6±0.79*^{c}	7.8 ± 0.88*^{c}	7.6±0.82^{c}	9.2 ± 0.46*^{b}	7.6±0.39^{b}	7.8 ± 0.27*^{c}	6.9±0.18*^{a}
6	2.4 ± 0.36	2.5*± 0.31	4.2 ± 0.36^{b}	4.5± 0.52^{b}	8.4 ± 0.38^{a}	6.9±0.81^{b}	9.0 ± 0.55^{a}	7.8±0.86^{b}	8.6 ± 0.33^{b}	7.2±0.65^{c}	9.0 ± 0.57^{a}	7.0±0.51*^{b}
7	2.4 ± 0.36	2.5*± 0.31	4.4 ± 0.22^{a}	5.1± 0.63^{a}	7.8 ± 0.22^{b}	8.7±0.95^{a}	8.2 ± 0.41^{b}	7.9±0.26^{a}	9.1 ± 0.41^{a}	8.0±0.28^{a}	8.9 ± 0.61^{b}	7.8±0.93*^{a}
8	2.4 ± 0.36	2.5*± 0.31	3.8 ± 0.32^{c}	3.5± 0.66^{c}	7.0 ± 0.36^{c}	5.1±0.64^{c}	7.8 ± 0.32^{c}	7.3±0.58^{c}	8.5 ± 0.64^{c}	7.4±0.43^{b}	6.2 ± 0.39^{c}	5.5±0.61^{c}

* Means with different, superscripts are significantly different (P ≤ 0.05).
a: highly significant difference, b: moderate significant difference, c: less significant different. (p<0.01)

Table 3.Mean NDV HI titer of broiler chickens vaccinated with different vaccination programs

Groups	Mean HI Titer of ND					
	7 days	14 days	21 days	28 days	35 days	42 days
1	4.0 ± 1.2*	2.1 ± 0.75	1.3 ± 1.1	1.1 ± 1.02	0.5 ±1.1	0.5 ± 0.9
2	2.8± 0.90	2.9 ± 0.6*	4.1 ± 0.91*	4.9 ± 0.13*	4.5 ± 0.2*	1.3 ± 1.2*
3	2.8± 0.90	3.3 ± 0.8*^{b}	4.9 ± 0.89*a	5.9± 1.01*a	7.5 ± 0.90*a	7.5± 0.48*a
4	2.8± 0.90	3.5 ± 0.35*^{a}	4.6 ± 0.71*b	5.1 ±1.1*b	5.9 ± 0.69*b	5.0± 0.66*c
5	2.8± 0.90	3.0 ± 0.74*^{c}	4.4 ± 0.99*c	4.9± 0.99*c	6.0 ± 0.76*b	6.0 ± 0.87*b
6	4.0 ± 1.2*	2.2 ± 0.36^{c}	4.4 ± 0.78b	4.8 ± 1.05b	5.0 ± 1.30b	6.0 ± 0.77b
7	4.0 ± 1.2*	2.4 ± 0.22^{b}	4.6 ± 0.82a	5.0 ± 1.4a	5.1 ± 0.77a	6.9 ± 0.89a
8	4.0 ± 1.2*	2.8 ± 0.32^{a}	4.2 ± 0.96c	4.9 ± 1.02c	5.0 ± 1.04b	5.9 ± 0.91b

* Means with different, superscripts are significantly different (P ≤ 0.05).
a: highly significantly different, b: moderate significantly different, c: less significant different. (p<0.01)

The ELISA results depicted in table 4 exhibit the highest titer after receiving live and inactivated vaccines in groups no. 3,4 and 5 at 14 days with mean titers 1538.4,1418.6and 1601 respectively. At 21 days the same groups were still giving the highest titer. While group no. 4 (Montanide ISA 71 adjuvanted group) at 28 days was showing a significant high titer among all groups with means titer of 3680, the group no. 3 (Montanide ISA 70VG adjuvanted group) could show significant high titer of 5130 and 8671 at 35 days and 42 days respectively. On the other hand, groups no. 6, 7 and 8 which received only the inactivated vaccines could show the highest serological response at 14 and 28 days with mean titers of 1601 and 1664; respectively.

Table 4. The Antibody response to inactivated combined IB+ND vaccines with different adjuvants using ELISA test in broilers.

Mean ELISA titer												
Age	**7 days post vaccination**		**14 days post vaccination**		**21 days post vaccination**		**28 days post vaccination**		**35 days post vaccination**		**42 days post vaccination**	
Groups	**Titer**	**C.V**	**Titer**	**C.V**	**Titer**	**C.V**	**Titer**	**C.V**	**Titer**	**C.V**	**Titer**	**C.V**
1	1538.4 ± 12.9	15%	145 ± 20.9	10%	176 ± 12.4	8%	198 ± 15.6	7%	179 ± 13.9	12%	263 ± 12.8	15%
2	1418.6 ± 17.1	18%	927.5 ± 19.8*	16%	1284 ± 17.9*	10%	2157 ± 17.4*	12%	1513 ± 22.5*	11%	1765 ± 19.9*	21%
3	1418.6 ± 17.1	18%	1538.4 ± 22.5*^{b}	15%	2430 ± 35.3*^{b}	33%	3730 ± 22.7*^{c}	10%	5130 ± 29.1*^{a}	18%	8671± 22.6*^{a}	15%
4	1418.6 ± 17.1	18%	1418.6 ± 23.9*^{c}	18%	2395± 26.4*^{c}	8%	3860± 23.3*^{a}	18%	4895± 24.1*^{b}	15%	7901± 21.5*^{b}	21%
5	1418.6 ± 17.1	18%	1601 ± 18.7^{a}	21%	2664± 24.7*^{a}	37%	3850± 22.1*^{b}	23%	4061± 21.3*^{c}	11%	7690 ± 25.4*^{c}	12%
6	1538.4 ± 12.9	15%	1430± 25.3^{b}	7%	1312± 21.1^{c}	33%	3312± 16.9^{a}	10%	2730± 15.7^{c}	21%	4800± 26.9^{b}	14%
7	1538.4 ± 12.9	15%	1240 ± 19.9^{c}	13%	1395 ± 20.3^{b}	8%	2430 ± 20.6^{b}	22%	2860± 19.6^{a}	14%	4680 ± 23.3^{c}	20%
8	1538.4 ± 12.9	15%	1601 ± 16.5^{a}	21%	1664 ± 19.7^{a}	17%	2395 ± 19.6^{c}	30%	2850± 22.7^{b}	!2%	5095± 20.4^{a}	9%

C.V: Coefficieance Variation,* Means with different, superscripts are significantly different (P ≤ 0.05).

a: highly significantly different, b: moderate significantly different, c: less significant different. ($p<0.01$)

Detection of IBV nucleic acid in vaccinated and challenged broiler chickens:

Real-time RT-PCR has been used for successful detection of IBV nucleic acid in vaccinated broilers as shown in table 5. Nucleic acid of IBV could first be detected at 3 days post vaccination only in the trachea of groups which received live vaccine either alone or with inactivated booster like in groups 2, 3, 4 and 5. During this time, IBV nucleic acid could not be detected in the kidnyes of vaccinated birds. On the other hand, IBV nucleic acid started to appear in the kidneys of these groups at 7 days post vaccination. At 10 days post vaccination, both trachea and kidneys were showing –ve results with rRT-PCR. Trachea and kidneys of all groups showed +ve reaction in the rRT-PCR at 7 days post challenge, while only kidneys could exhibit +ve RT-PCR re-activities at 10 days (Table 6). At 7 days post challenge group no. 4 that took live vaccine with experimental MEVAC IB+ND ISA 71 showed good protection in trachea compared with group no. 2 that took live vaccine only. Birds In group # 7 only vaccinated with the experimental MEVAC IB+ND ISA 71, exhibited much more protection if compared with group no. 2 (primed only with live vaccine). Kidneys of birds in group no. 4 could show the best protection if compared to group no.2 at 7 days post challenge (table 6).

Table 5: Detection of IBV nucleic acid using RT-PCR in vaccinated Broiler groups.

Group No.	3 days p.v.		7 days p.v.		10 days p.v.	
	Trachea	Kidney	Trachea	Kidney	Trachea	Kidney
1	-ve	-ve	-ve	-ve	-ve	-ve
2	+ve (35)	-ve	+ve (37)	+ve (32)	-ve	-ve
3	+ve (35)	-ve	+ve (37)	+ve (32)	-ve	-ve
4	+ve (34)	-ve	+ve (37)	+ve (32)	-ve	-ve
5	+ve (34)	-ve	+ve (36)	+ve (30)	-ve	-ve
6	-ve	-ve	-ve	-ve	-ve	-ve
7	-ve	-ve	-ve	-ve	-ve	-ve
8	-ve	-ve	-ve	-ve	-ve	-ve

Table 6: Detection of IBV nucleic acid using rRT-PCR in challenged Broiler groups:

Group No.	3 days p.C.		7 days p.C.		10 days p.C.	
	Trachea	Kidney	Trachea	Kidney	Trachea	Kidney
1	-ve	-ve	-ve	-ve	-ve	-ve
2	+ve (25)	-ve	+ve (22.51)	+ve (26)	-ve	+ve (31)
3	+ve (27)	-ve	+ve (31.21)	+ve (28)	-ve	+ve (29)
4	+ve (28)	-ve	+ve (34.52)	+ve (30)	-ve	+ve (28)
5	+ve (27)	-ve	+ve (31.43)	+ve (25)	-ve	+ve (30)
6	+ve (26)	-ve	+ve (36.67)	+ve (26)	-ve	+ve (29)
7	+ve (27)	-ve	+ve (37.59)	+ve (27)	-ve	+ve (29)
8	+ve (25)	-ve	+ve (30.21)	+ve (27)	-ve	+ve (30)

-ve: negative result, +ve: positive result

DISCUSSION:

Newcastle Disease (ND) and infectious bronchitis (IB) are important diseases in the poultry industry and cause great losses (King & Cavanagh, 1991; Tu et al., 1998).IBV is one of the most important respiratory diseases that affects chickens of all ages and characterized by severe loss of production and egg quality in mature hens. Some strains cause nephritis in young birds and others are occasionally reported to be associated with enteritis (Gorgyo et al., 1984).Newcastle disease (ND) is one of the most infectious ,highly contagious, fatal viral diseases of chickens, characterized by respiratory, digestive, and nervous symptoms (Mishra et al., 2000).

Control involves the use of biosecurity procedures and vaccination (Mayahi et al., 2013), which is routinely used throughout the intensive poultry industry .Both live attenuated and oil emulsion inactivated vaccines are available(OIE, 2013). In our study we used a live attenuated IBV vaccine and live NDV vaccine for priming of broiler chickens at one-day old in groups no 2, 3, 4 and 5. Then at 7 days old the broiler chickens of all groups were boosted with the inactivated vaccines, except for groups no. 1 and 2 (Table 1).

Live vaccines, attenuated by serial passage in chicken embryos or by thermal heat treatment, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines (OIE, 2008).The choice of vaccine strains should be based on prior information as to which antigenic types are prevalent in the particular county or region. The vaccines used most frequently are based on Massachusetts strains (which is serotype of world-wide importance), example of these are M41, Ma5, H52 and H120. Other monovalent vaccines for regional use are also available as strain D274, D1466 and 4/91 in European countries .Live vaccine strains from other parts of the world should not be used or introduced if prevailing endemic strains are of a different serotype or genetic lineage (Ignjatovic and Sapats, 2000). The live vaccines being used here for Priming to the inactivated vaccines, is IB Primer which contains H120 as classical strain and D274 as variant strain in parallel with hitchner strain of NDV shown in (Table 1).whereas DHINAKAR RAJ &. JONES (1997)appeared that, following vaccination of chickens with live IBV vaccine, cross-reactive cellular immune responses occur that vary in magnitude with the strain of IBV used for in vitro stimulation.

Inactivated vaccines are injected and a single inoculation does not confer protection unless preceded by one or more live IBV priming vaccinations. Both types of vaccines are available in combination with Newcastle disease vaccine; in some countries inactivated multivalent vaccines are available that include two to three IBV antigens or Newcastle disease, infectious bursal disease, reovirus and egg-drop syndrome 76 viral antigens(OIE, 2008).

Although live vaccines are generally inexpensive, easy to administer and give high titers but the titers are maintained for shorter period of

time. The oil based vaccines are little expensive but give good titers, which are maintained over a long period of time (Burgh and Siegel., 1978). Both the commercial and experimental MEVAC IB+ND are inactivated combined trivalent vaccines of infectious bronchitis virus containing Eg/11539F (IB Classical), Eg/1212B (IB Variant type 2) and NDV/Chicken/Egypt/11478AF/2011. They are all locally isolated strains in Egypt. In addition to the imported vaccine Nobilis® IB multi+ND+EDS inactivated combined vaccine for chickens against IB, Newcastle Disease and Egg Drop Syndrome containing serotype Massachusetts M41 (IB Classical) , D207/ strain D274 and related strains (IB Variant type 2) and NDV.

The enhancement of cross protection against isolates belonging to antigenically different serotypes may occur particularly if revaccination is carried out at approximately 2 weeks of age, using a licensed IB vaccine of a different serotype than the one used initially (Maloet al., 1998). In broilers vaccination against IB is usually carried out at day old .The protection provided by a single vaccination may not be enough to cover for the entire production period. Application of a second IB vaccination may well be beneficial in such situations, not only to prolong the duration of the protection obtained but also to broaden the spectrum of such protection (Maloet al., 1998).That is why we used both classical and variant strains as initial vaccination and boosted with inactivated vaccine contains also both classical and variants. (Table 1)

Inactivated vaccines are prepared by treating the virus with various chemicals such as formalin, beta propiolactone and phenol or with physical means. These oil-emulsion vaccines are prepared by combining appropriate quantities of mineral oil, emulsifier and desired antigen in various mechanical devices to incorporate aqueous antigen within the surfactant covered particles (Mahboob et al., 1999). Parentally inoculated antigens contained in oil-emulsion adjuvant generally stimulate higher and more persistent antibody titers than equivalent amounts of antigen inoculated without adjuvant (Stone et al., 1978). This is agreed with our study as there was an increase in HI titer in all groups (3-8) at 14 days, except for control no. 1 and group no. 2 (didn't receive an adjuvanted vaccine),but with significant increase in group no. 7 which received experimental MEVAC IB+ND ISA71 alone and group no. 4 which received live priming with Hitchner-IB Primer vaccine followed by vaccination with experimental MEVAC IB+ND ISA70 (Table 2).the antibody response of the chickens to NDV showed that groups 3 and 7 at 21 days were giving the highest titers 4.9 and 4.6 log2, these Groups continued to give a high titer in 28, 35 and 42 days old .Maternally derived antibodies (MDA) can provide protection against IBV, but they are short-lived. Presence of MDA has no adverse effect on the efficacy of live IBV vaccines administered at one-day of age (Cook et al, 1991b).

Our ELISA results depicted in table 4 exhibit the highest titer after receiving live and inactivated vaccines in groups no. 3, 4 and 5 at 14 days with mean titers 1538.4, 1418.6 and 1601 respectively. At 21 days the same groups were still giving the highest titer. While group no. 4 (Montanide ISA 71 adjuvanted group) at 28 days was showing a significant high titer among

all groups with means titer of 3680, the group no. 3 (Montanide ISA 70 adjuvanted group) could show significant high titer of 5130 and 8671 at 35 days and 42 days respectively. On the other hand, groups no. 6, 7 and 8 which received only the inactivated vaccines could show the highest serological response at 14 and 28 days with mean titers of 1601 and 1664; respectively. Chickens develop a good humoral response to IBV infections, measurable by enzyme-linked immunosorbent assay (ELISA), haemagglutiation inhibition (HI) or VN tests (De Wit et al, 1992);. However, there is a lack of correlation between titers of circulating antibodies and resistance to infection (Alexander,1977).

Immunoglobulin G (IgG), the major circulating, is the antibody detected by HI and an ELISA developed to measure it is more sensitive (Mockett&Darbyshire, 1981). Anti-IBV IgG can be detected as soon as four days pi, reaches a peak at about 21 days but can remain in high titer in the serum for many weeks (Mockett and Darbyshire, 1981). This is the antibody measured in serological tests to monitor IBV infections or vaccine uptake .IgM present only transitorily after infection, reaches peak concentrations about 8 days after IBV infection and levels then decline (Mockett and Cook, 1986). Although an IgM-specific ELISA has been shown to be useful in the diagnosis of recent infections (Martins et al., 1991).

IGNJATOVIC & GALLI (1995) monitored antibody response in chicks vaccinated with either live or inactivated IBV against S1, S2 and N proteins that elicited similar titers of antibodies following vaccination with live IBV, whereas the M glycoprotein elicited significantly lower titers. Time of appearance and the course of development of the S1, S2 and N ELISA antibodies were similar, being first detected 2 weeks after vaccination and coincided with appearance of virus neutralizing antibodies. The M antibodies were first detected 4 weeks after vaccination. S1, S2, and N antibody titers were significantly higher in chicks vaccinated at 14 days of age than in chicks vaccinated at either 1 or 7 days of age, and reached maximum levels 4 weeks after the second vaccination. Vaccination with inactivated virus induced significantly lower antibody titers and at least three vaccinations were necessary for induction of S1, S2, N and M antibodies in all chicks.

Our results revealed the antibody response of the chickens to different vaccination programs using M41& D 274 antigen (table 2) that the mean HI antibody titer to IBV started to increase after 7 days in groups 2,3,4 and 5 which received live vaccination at one day old. At 14 days (7 days after boosting with inactivated vaccine) there was an increase in HI titer in all groups (3-8), except for control no. 1 and group no. 2, but with significant increase in group no. 7 which received experimental MEVAC IB+ND ISA71 alone and group no. 4 which received live priming with Hitchner-IB Primer vaccine followed by vaccination with experimental MEVAC IB+ND ISA70. The highest titer was noticed in group no. 3 at 28 days with a titer of 9.5 log2. Then the titers started to decrease at 42 days to range between 5 log2 and 8 log2. Momayez et al. (2008) explored the level of antibody response was measured by HI test was found to be protective and compatible with the titer of HI recommended by OIE and also compatible with those of challenge test,

therefore this test can be used for the potency control of the oil-emulsion killed IB vaccine.

In regarding the challenge test at 21 days post-last-vaccination, the signs appear 5 days post-infection as mild respiratory manifestation and at one week post infection the birds' kidneys have gross lesions (nephritis and nephrosis) as reviewed by DHINAKAR & JONES (1997).Replication of IBV in the respiratory tissues causes characteristic, but not pathognomonic signs such as gasping, coughing, tracheal rales and nasal discharge. Occasionally, puffy, inflamed eyes and swollen sinuses may be seen. In uncomplicated cases these signs last for only 5 to 7 days and disappear within 10 to 14 days. The affected chickens also appear depressed, and feed consumption and weight gain are significantly reduced from 3 days after infection. The upper respiratory tract is the main site of IBV replication, following which a viremia occurs and the virus gets widely disseminated to other tissues as kidney and oviduct.

During the clinical phase of the disease, maximum virus titers are recorded in the trachea between 5 and 10 days p.i. (Cook, 1968.).In our study, Real-time RT-PCR has been used for successful detection of IBV nucleic acid in vaccinated broilers as shown in table 5. Nucleic acid of IBV could first be detected at 3 days post vaccination only in the trachea of groups which received live vaccine either alone or with inactivated booster like in groups 2, 3, 4 and 5. During this time, IBV nucleic acid could not be detected in the kidneys of vaccinated birds.

On the other hand, IBV nucleic acid started to appear in the kidneys of these groups at 7 days post vaccination. At 10 days post vaccination, both trachea and kidneys were showing –ve results with rRT-PCR. Trachea and kidneys of all groups showed +ve reaction in the rRT-PCR at 7 days post challenge, while only kidneys could exhibit +ve RT-PCR re-activities at 10 days (Table 6).Although even those strains of IBV considered primarily affecting the respiratory tract such as M41 can occasionally cause kidney damage, nephro-pathogenicity has been associated only with certain strains. Greater virulence of the virus for the kidney was first detected 7-10 days post- infection (DHINAKAR &JONES, 1997). As well as the authors recorded that genetic differences in infected chicks have variation in susceptibility to nephritis, with light breeds being more susceptible than heavy breeds.

At 7 days post challenge group no. 4 that took live vaccine with experimental MEVAC IB+ND ISA 71 showed good protection in trachea compared with group no. 2 that took live vaccine only. Birds In group no. 7 only vaccinated with the experimental MEVAC IB+ND ISA 71, exhibited much more protection if compared with group no. 2 (primed only with live vaccine). Kidneys of birds in group no. 4 could show the best protection if compared to group no.2 at 7 days post challenge (table 6).

Humoral antibodies seemed to protect the tracheal epithelium following challenge. Presences of high titers of humoral antibodies correlate well with the absence of virus recovery from kidneys and genital tract (Yachida et al, 1985). IBV-specific antibodies probably prevent the spread of virus by viraemia from the trachea to other susceptible organs such as the kidneys and oviduct (DHINAKAR and JONES, 1997).

Antigen-specific proliferation of T-lymphocytes in IBV-infected or

vaccinated chickens has been demonstrated by Timms&Bracewell (1983). In some chickens, a positive correlation between lymphoproliferative responses and resistance to challenge has been shown (Timms&Bracewell, 1981). Monoclonal antibodies (Mab) that distinguish between chicken T lymphocytes have been described (Chan et al., 1988). The CD4 and CD8 antigens are found on two main populations of T-cells, T-helper (Th) and T-cytotoxic / suppresser (Tc/S) cells, respectively. Janse et al. (1994) contended that local immunity to IBV in the trachea is mediated by T-cells. CD4 and CD8 cells were shown in sections of trachea and lung of chickens infected with IBV. However, it is not clear which of these cells are more important in virus clearance, since Janse et al. (1994) found an increase in CD4 cells, while Dhinakar Raj & Jones (1996a) found higher proportions of CD8 cells. The differences may be related to the strains of IBV used in infection (DHINAKAR and JONES, 1997).

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