Studies on proventriculitis in Broilers with molecular characterization to its viral causes

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Abstract: Infectious proventriculitis syndrome is at times a serious and ongoing problem causing significant financial losses. From the obtained results in the present study, it could be concluded that proventriculitis can be transmitted by oral inoculation of homogenates produced from proventriculi of birds with proventriculitis. Experimentally inoculated broilers were seroconverted to ARV, IBV and IBDV. I-FAT against ARV, IBV and IBDV detected intra-cytoplasmic staining in the proventricular glandular epithelial cells of experimentally inoculated broilers. ARV, IBV and IBDV were identified in proventricular homogenate inoculated broilers by RT-PCR. S1 sequence analysis of examined ARV isolates revealed a higher degree of similarity with published Egyptian isolates than that obtained when compared with international strains. S1 sequence analysis of examined IBV isolates revealed their close relatedness to Mass serotype. Nested PCR followed by RFLP and sequence analysis of HVR of VP2 gene revealed that the examined IBDV isolates related to vvIBDV. [Journal of American Science 2010; 6(9):582-592]. (ISSN: 1545-1003).

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1. Introduction

Proventriculitis is the common name given to a clinical condition of processed broiler chickens, especially in those birds processed at 4 to 5 weeks of age, characterized by proventricular enlargement along with weakness of the proventriculus and gastric isthmus. This condition results in tearing of the intestinal tract during mechanical evisceration and subsequent contamination of the carcass with intestinal contents. Proventriculitis of broilers has been associated with intestinal fragility, poor feed conversion, stunting syndrome, and passage of undigested feed (Apple et al., 1991). Losses due to associated production problems are compounded by a resulting increase in the number of reprocessed carcasses, downgrades, and condemnation, which cost poultry industry millions of dollars annually (Leonard and Schmittle, 1995).

Proventriculitis has become a food safety concern because the contents of the ruptured proventriculus are a major source of feces contamination of processed chicken carcasses, resulting in an increased possibility of contamination with human pathogens (Thayer and Walesh, 1993). A number of viral, bacterial, and fungal agents (Brugh and Wilson, 1986; Tsai et al., 1992; Lenz et al., 1998; Huff et al., 2001; Schulze and Heidrich, 2001 and Yu et al., 2001) have been isolated from birds with proventriculitis. Some of these infectious agents, along with other factors including feed texture and fiber levels (Riddell, 1976), biogenic amines (Barnes et al., 2001), mycotoxins (Cullen et al., 1988), and high levels of dietary copper sulfate (Bayyari et al., 1996), have been shown to cause proventriculitis or proventricular enlargement.

From the above mentioned data, our study was planed for molecular characterization of the common viruses which accompanied with this clinical condition as well as to study the changes in proventriculus and other organs.

2. Material and Methods

Field samples:

Proventriculi were collected from 25 commercial broiler chicken flocks between 3 to 7 weeks of age. These flocks were suffering from mortality rate varies between 1 to 10%; poorly feathered and pale chicks, reduced growth and/or excessively uneven weight distribution at slaughter age. At necropsy of chickens with lower live weight than expected, the proventriculus was enlarged with thick wall and the proventricular isthmus was wide and flaccid.

Preparation of proventriculus homogenate:

It was carried out according to Bayyari *et al.* (1996) as follows: proventriculi were washed in sterile phosphate-buffered saline (PBS) three times and then diluted 1:1 w/v in PBS containing antibiotic penicillin and stryptomycine and homogenized in sterile mortar with sterile sand and pestle under

aseptic condition. The homogenates were frozen and centrifugation. The clear supernatants were aliquoted and stored at -80°C until used.

Experimental chicks:

Four hundred and fifteen, one-day-old broiler chicks (Ross) were used in this experiment. Chicks were kept in separate metal cages under hygienic measures and feed on adequate commercial broiler ration and water. 25 chicks were used for determination the time of maternal derived antibodies (MDA) waning. At 3 weeks of age, 390 chicks were divided into 26 groups (15 chicks /group). The first 25 groups of them were used for experimental induction of proventriculitis; the last group of chickens was left as blank control.

Histopathological examination:

Samples from proventriculus, bursa, thymus, and spleen were collected from experimentally infected chickens for histopathological examination according to the method of Pantin-Jackwood et al. (2005).

Serology: Sera were collected at 2 and 3 weeks post infection (PI)) and tested individually for presence of antibody against IBDV, IBV and ARV by ELISA test thawed three times; sediment was removed by (IDEXX Laboratories) and against ND virus by HI test.

Indirect fluorescent antibody technique (I-FAT):

The frozen proventriculus tissues used for section preparation were kept at -80°C until cut using freezing microtome with thickness of 8 microns. Sections were collected on glass slides and fixed with cold acetone directly for 20 minutes then marked and kept at -80°C till examined for the presence of IB, IBD and ARV using reference antiserum against ARV, IB and IBD as the primary antibody and goat anti-chicken IgG FITC conjugated as the secondary antibody. Test was carried according to method described by Guy and Barnes (2003).

RNA extraction:

Five different proventriculus samples were used for RNA extraction from ARV, IBV and IBDV. RNA extraction was performed according to the protocol described by the manufacturer's RNA extraction kit reagents (QIAamp® Viral RNA Mini kit).

Oligonucleotide primers: Synthesized by Metabion Company, Germany as described in the following tables:

- **Oligonucleotide primers for amplification of S1 gene of ARV by RT-PCR:**

Primer	Sequence (5`-3`)	Reference
MK87	GGTGCGACTGCTGTATTTGGTAAC	Shapouri et al. (1995)
MK88	AATGGAACGATAGCGTGTGGG	Shapouri et al. (1995)

These primers were published by Xie et al. (1997) to flank 532 bp DNA sequence containing the S1 gene.

- **Oligonucleotide primers for amplification of S1 gene of IBV by RT-PCR:**

Primer	Sequence (5`-3`)	Reference
U1	ACTGAACAAAAGACACGACTT	Jungherr et al. (1956)
L1	CCACCAGAAACTACAAACTG	Jungherr et al. (1956)

These primers were published by Xu et al. (2007) based on the cDNA sequences of genomic segments S1 of IBV M41 from GenBank (GenBank Accession number M21883), a pair of primers U1/L1 was designed and used to amplify the S1 glycoprotein gene (1596 bp) of IBV isolates.

- **Oligonucleotide Primers for amplification of VP2 gene and hypervariable region (HVR) of VP2 gene of IBDV for nested PCR:**

Primer	Sequence (5`-3`)	Position	reference
VP2 upstream	GCGATGACAAACCTGCAA GAT	93-114 bp	Bayliss et al. (1990)
VP2 downstream	AGGTGGGAACATGTGGAGAC	1470-1490 bp	Bayliss et al. (1990)
HVR upstream	TCACCGTCCTCAGCTTAC	587-604 bp	Bayliss et al. (1990)
HVR downstream	TCAGGATTTGGGATCAGC	1212-1229 bp	Bayliss et al. (1990)

These primers were published by Dormitorio et al. (1997) to flank the hypervariable region of VP2 gene amplifying a product of 643 bp in length.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay:

RT-PCR assay was performed using Ready-To-Go RT-PCR Beads (Amersham, USA). Each bead was resuspended in 40 µl DEPC treated water, then adding 1/2 µl of each primer forward and reverse and 3 µl RNA. Complete the mixture with DEPC treated water to 50 µl (reaction volume) and covered with 1 µl mineral oil.

RT reaction was denaturated at 95^{o}C for 5 minutes and then put on ice (only for ARV and IBDV). The samples were placed on thermal cycler at 42^{o}C for 30 minutes, then heated at 95^{o}C for 5 minutes as initial denaturation. Thirty nine PCR cycles were consisting of denaturation (92^{o}C for 1 minute), anealing (50^{o}C for 1 minute) and extension (72^{o}C for 1 minute). The final extension was conducted at 72^{o}C for 10 minutes.

After the end of PCR run, the product was analyzed with agarose gel electrophoresis using 1% agarose gel stained with ethidium bromide.

Nested PCR for IBDV:

Nested PCR was used to confirm the specificity of the RT/PCR reaction using internal primers inside the PCR product amplifying the hypervariable region (HVR) which was used for molecular subtyping of IBDV using restriction endouclease digestion (RFLP).

5 µl of PCR product of first assay were used as template of second reaction. The nested PCR reaction was prepared from PCR product, dNTPs, 10 x buffers, Taq DNA polymerase, forward and Reverse primers, 25mM $MgCL_2$ and DEPC treated water.

The reaction covered with 1 µl mineral oil then the samples were placed on thermal cycler at 95^{o}C for 5 minutes as initial denaturation. Thirty nine PCR cycles were consisting of denaturation (92^{o}C for 1 minute), anealing (50^{o}C for 1 minute) and extension (72^{o}C for 1 minute). The final extension was conducted at 72^{o}C for 10 minutes.

Analysis of amplified PCR product by electrophoresis: The method was carried out according to Sambrook et al. (1989).

Restriction fragment length polymorphism (RFLP) for IBDV:

The molecular subtyping was carried out using BstNI and SspI as previously described by Jackwood and Sommer (1998).

purification of PCR product:

QIAquick PCR Purification Kits used for direct purification of PCR products from amplification reactions and DNA cleanup from other enzymatic reactions as described by manufacturer manual of Qiagen, Germany, PCR Purification kit.

Sequence of PCR product:

All sequencing reactions were carried out at the JenaGen GmbH Diagnostik-Gentechnik-Biotechnologie, Thüringen, Germany. The obtained nucleotide sequences were analyzed using DNASTAR software.

3. Results and Discussion

The prevalence of proventriculitis among broiler chicken flocks in Egypt was carried out through field surveillance. In first experiment, twenty five proventriculus samples were collected from commercial broiler flocks in different governorates of Egypt. The flocks were generally characterised by poor feed conversion. Same clinical signs were reported by Goodwin *et al*. (1996); Huff *et al*. (2001); Reece (2002); Guy and Barnes (2003); Hussein *et al*. (2003); Shaban (2004) and Ali (2009). Necropsy of chickens with lower live weights than expected and/or those voiding poorly digested or mucoid covered feces revealed a high incidence of increased size of the proventriculus. The proventricular isthmus was wide and flaccid. The proventricular wall was thickened, with white lobular patterning upon sectioning. The lumina of some proventricular lobules were distended with clear fluid that exuded when the proventricular wall was cut. Our findings are in agreement with previous records (Goodwin et al. 1996; Huff et al., 1997; Huff et al., 2001; Reece, 2002; Guy and Barnes, 2003; Hussein et al., 2003; Shaban, 2004; and Ali, 2009).

In first section of the second experiment showed the results of serological follow up of maternally derived antibody (MDA) against ARV, IB and IBD in 3 day-old broiler chicks by ELISA test. The maximum ELISA antibody titers against IBDV; IBV and ARV were detected at 3 day old, subsequently declined till becoming negative (ELISA titers less than 395) at 21 day old. Also the highest HI maternally derived antibody titers against NDV at 3 day old then declined gradually till reach zero at 21 days old. The results agreed with that obtained by Ahmed and Akhter (2003) who found that maternal antibodies in unvaccinated chickens persisted in chicks up to 21 days as determined by ELISA with complete decay by 28 and 35 days.

Second section of the second experiment showed the results of experimental induction of proventriculitis in 25 groups of 21 day-old chicks each inoculated orally with one ml of proventricular homogenates. No characteristic signs were observed after inoculation of the proventricular homogenates; but general signs such as illness and depression were observed after one week post inoculation in most infected chicken groups (20 groups). Diarrhea was

observed in 10 groups of them. No signs were observed in 5 groups in addition to control group. Similar findings were recorded by Skeeles *et al*. (1997).

Necropsy of chickens with lower body weights than controls or those voiding poorly digested feces at 1, 2 and 3 weeks PI revealed that the proventriculi were enlarged and the outer surface was mottled with gray-white plaques due to individual glands seen from the serosal surface, thickening of the mucosa and flattening of the proventricular glands as shown in Photo (1). Similar results supporting our findings were recorded by Goodwin et al., 1996; Goodwin and Hafner, 1997; Huff et al., 1997; and Skeeles et al., 1997, the later were able to reproduce proventriculitis in both SPF leghorns and IBDV maternally immune commercial chickens, Dormitorio et al., (2000); Nighot and Kolte, (2000); Skeeles and Newberry,(2000); Dormitorio et al. (2001b); Guy and Barnes, (2003); and Reece, (2003)
Serological testing indicated that 80% out of 25 experimentally inoculated groups had positive antibody titer against ARV, IBV and IBDV by ELISA. For ARV and IBV, 8 groups (32%) out of 25 were positive reactor for each infection, while 10 groups (40%) out of 25 were positive for IBDV through ELISA test. The recorded results of HI proved the absence of HI-titers against NDV from all examined groups. Pantin-Jackwood et al., (2005) reported negative seroconversion for ARV, NDV, MG and MS at 7 and 14 days PI in commercial broilers that received proventricular homogenates. These birds were positive for IBDV and IBV at both time points.

The pathogenesis of TVP is poorly understood. Based on damage observed in glandular epithelium, Goodwin *et al*. (1996) and Goodwin and Hafner (1997) suggested that clinical effects observed in this disease likely were due to destruction of proventricular pepsinogin-and hydrochloric acid-secreting glandular epithelial cells.The histopathology findings in the infected proventriculi of chicks experimentally inoculated with proventriculi of the present study support that hypothesis. Microscopic changes in proventriculi revealed necrosis of glandular epithelium resulting in a net loss of glandular tissue. The loss of glandular epithelium likely would result in loss of function, as suggested by Goodwin *et al*. (1996) and Goodwin and Hafner (1997)**.**

Histopathological finding in the collected bursae, thymuses and spleens of experimentally inoculated chicks with proventricular homogenates characterized by progressive and severe lymphoid necrosis and depletion (photo to show the histopathological finding). Similar lesions were reported by Sharma *et al*. (1989).

From the above mentioned results transmissible proventriculitis (TP) was successfully reproduced in chickens using inoculums derived from proventriculi collected from proventriculitis affected broiler chickens (Guy and Barnes, 2003). Lesions produced in experimentally infected chickens were identical with those in naturally occurring TP cases so the TP inoculums were infectious and transmissible. Since the proventricular homogenates were mixed with antibiotics to remove bacteria, it is believed that the TP agent(s) is a virus. Similar trials with the same results were carried out by Huff *et al*. (2001) and Guy and Barnes (2003). Also the results suspect that we are dealing with ARV, IBV and IBDV related isolates.

In third experiment, the results of I-FAT that applied on frozen proventriculi collected from chickens showing positive in ELISA test proved the presence of ARV, IBV and IBDV antigens as positive staining in the cytoplasm of the glandular epithelial cells. There were 17 groups (85%) out of 20 experimentally inoculated groups had positive staining against ARV, IBV and IBDV by I-FAT. Our results revealed that the numbers of positive samples monitored by I-FAT were 6/20 (30%); 5/20 (25%); and 8/20 (40%) for ARV; IBV and IBDV respectively; at 3 wks PI of broilers with proventricular homogenate.

Our work confirmed that, immunofluorescent was preferred for virus detection, because it was sensitive. It could be performed very rapidly on a large number of specimens. These results were similar to that recorded by Gardner (1977); Mcferran *et al*. (1980); Allan *et al*. (1984); Yagyu & Ohta (1990); Nakamura *et al*. (1991); Nunoya *et al*. (1992); Cruz-Coy *et al*. (1993); Chen *et al*. (1996) and Dhinakar Raj and Jones (1996).

Various methods have been developed for the diagnosis of viral infections, such as virus isolation, electron microscopy, FAT, ELISA, immunodiffusion, viral neutralization and immmunoblot (Rosenberger, 1989; Lukert and saif, 1991; Wu et al., 1992; and Liu et al., 1994). However, they suffer disadvantages, such as being time consuming, labor intensive, expensive and of low sensitivity (Wu et al., 1992).

The polymerase chain reaction (PCR) method for *in vitro* amplification of target gene sequences (Saiki et al., 1985) has been applied as a rapid diagnostic tool for detection of a range of avian viral pathogens (Lee et al., 1992; Kwon et al., 1993; and Xie et al., 1997). This method is not only more rapid but also is more sensitive and specific than other diagnostic procedures.

The forth experiment showed the results of molecular characterization of ARV, IBV and IBDV antigens by RT/PCR.

Based on the published sequence data of the S1 gene of the avian reovirus S1133 strain by Shapouri *et al*. (1995) the primers were designed to flank a 532 bp DNA sequence containing the S1 gene by RT/PCR. In the present study, all five examined field isolates were positive in the reovirus RT-PCR, with all positive products consisting of the expected 532 bp DNA band (Photo 2). The same results were obtained by Xie *et al*. (1997); Liu *et al*. (1999) and Caterina *et al*. (2004). Our local isolates were amplified for S1 gene for confirming the isolated samples, and also using the product of RT/PCR for sequencing and understanding the epidemiology and evolution of ARV. The nucleotide sequences of S1 fragments of ARV isolates consisted of 377 bp for isolates 1 and 2 and 273 bp for isolate 3.

Comparison between our three local ARV isolates with two Egyptian ARV isolates revealed that Isolates 1 and 2 had very high similarity 98.1% with isolate 57663 where isolate 3 showed high similarity 96.7% with isolate 57665. On other hand, the three examined local isolates showed high identity percent 93%-96% when compared with ARV international strains and isolates (Fahey-Crawley, S1133, 1733, 176, YJL, HB06, G-98, T-98, B-98 and P100) but in case of strain 138, they showed low similarity 77%-80% (Fig 1). Similar results were obtained by Saif El-Nasr (2008).

Based on the cDNA sequences of genomic segments S1 of IBV M41 from GenBank, a pair of primers U1/L1 was designed and used to amplify the S1 glycoprotein gene (1596 bp) of IBV isolates by RT/PCR. In our present study, all five examined isolateswere positive in the IBV RT-PCR, with all positive products consisting of the specific DNA band of 1596 bp (Photo 3). The same results were obtained by Xu *et al*. (2007). Our local isolates were amplified for S1 gene for confirming the isolated samples, and also using the product of RT/PCR for sequencing and understanding the epidemiology and evolution of IBV. The nucleotide sequence S1 gene of IBV isolates consisted of 1480 bp for isolates 1 and 2 and 1590 bp for isolate 3. Comparison between our three local IBV isolates with each other exhibited very high nucleotide similarity 97.6%-99.5%. Also, they showed very high nucleotide similarity 97.5%-99.9% when compared with Egyptian IBV (Egypt-F-03). The three examined local IBV isolates showed high identity percent 94%-98% with IBV international strains and isolates (H120, Ma5, M41, HN9604, W93, LKQ3, IBN and Beaudette US) but in case of strain GX1-98, they showed very high nucleotide similarity 97.4%-99.9% (Fig 2). From these results, we concluded that S1 sequence analysis of examined IBV isolates revealed their close relatedness to Mass serotype. The same results were obtained by Abdel-Moneim *et al*. (2006).

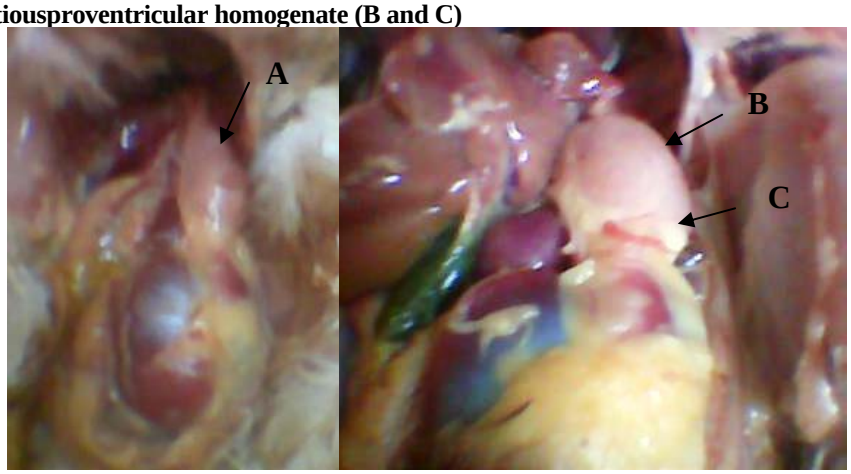
Based on the published sequence of IBDV Cu-1 strain by Bayliss *et al*. (1990), the primers were designed flanking the 5` and 3` conserved regions of VP2 for amplification of the full length VP2 gene by RT/PCR. As previous report by Liu *et al*. (1998) mentioned that nested PCR was of high sensitivity at least 100 times greater than RT/PCR. It was used in this study for more confirmation of the RT/PCR product of VP2 full gene. Internal primers were used that were published by Dormitorio *et al*. (1997) flanking the HVR of VP2 gene for amplification of a 643 bp using nested PCR. The results of this study demonstrated that the HVR for all five examined isolates were of identical size (Photo 4). The same results were obtained by Liu *et al*. (1994 and 1998) and Dormitorio *et al*. (1997**).** Nested PCR followed by RFLP was utilized a rapid and reliable differentiation tool for subtyping of the local isolates depending on the HVR of VP2 gene. The nested PCR products (643 bp) were digested with two restriction enzymes BstNI and SspI to distinguish between classic, variant and very virulent strains (Jackwood and Sommer, 1999). The BstNI enzyme could not differentiate between the five isolates as they remained undigested at 643 bp similar to that of the control positive vvIBDV isolate while the control positive classical IBDV isolate produced 4 bands at 210 bp, 171 bp, 151 bp and 110 bp.

The SspI enzyme digestion of 643 bp of the examined five IBDV isolates could not differentiate between the five isolates as they had similar RFLP profile of 2 fragments of length 390 bp and 253 bp similar to that obtained by the control positive vvIBDV isolate. On the other hand the control positive classical IBDV isolate did not have restriction sites for this enzyme. According to Lin *et al*. (1993) and Ture *et al*. (1998), the RFLP profiles of the five IBDV isolates with SspI indicate that all of them related to the vvIBDVs (Photo 5). Also our results was in the view of the results obtained by Jackwood and Sommer (1999) as they reported a new molecular group (gp 6) and used SspI restriction enzyme to predict vvIBDV. The obtained RFLP pattern revealed that no vvIBDV was obtained in molecular groups 2, 3 and 4 while within group 6 50% were SspI positive with a flock history supporting the vvIBDV phenotype. Our local isolates were amplified for HVR of VP2 gene for confirming the isolated samples, and also using the product of nested PCR for sequencing and understanding the epidemiology and evolution of IBDV. The nucleotide

sequence IBDV-HVR isolates consisted of 536 bp for four local isolates. Comparison between our four local IBDV isolates with each other exhibited very high nucleotide similarity 96.6%-99.1%. Although these isolates revealed 90.5%-100% identity when compared with published Egyptian IBDV isolates (Giza-2008, Kal-2001 and Giza-2000), Giza-2000 (vvIBDV) showed nearly complete identity 100%-98.9% with our isolates 1 and 3 respectively. The four examined local IBDV isolates showed identity percent 90%-93.5% when compared with IBDV international strains (Cu-1, STC, PBG-98, D78, VarA and Del E) but in case of strains OKYM and KS (vvIBDVs), they showed very high similarity 95.5%-98% (Fig 3). From these results, we concluded that the examined IBDV isolates related to vvIBDV. The same result obtained by Abd El-Moaty, 2004 Abd El-Moaty (2004). These results lead us to believe that possibly two or more viruses can infect and replicate in the proventriculus, causing a similar syndrome.

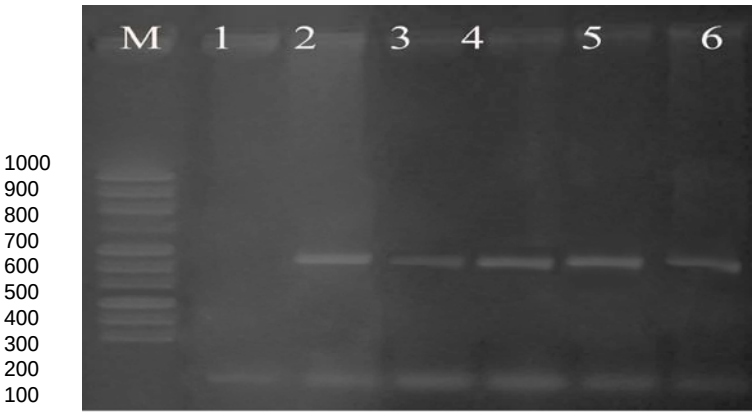
Because of the complexity and interaction of the many causative factors, prevention of proventriculitis may not be accomplished by a single vaccine. However, control of the infectious viral agents may decrease the incidence and/or severity of this problem. So, it's strongly recommended to use vaccines prepared from local field outbreaks after complete antigenic and genetic studies to put database for our vaccination programs.

Photo (1): Photographs of proventriculi from broiler chickens 2 wk. PI with saline (A) or with infectiousproventricular homogenate (B and C)



A) Normal proventriculus
B) Enlarged proventriculus
C) Dilated isthmus

Photo (2): Electrophoretic pattern of RT-PCR assay of S1 gene of ARV



Lane M showed 100 DNA ladder
Lane 1 showed no amplification in negative control sample.
Lanes 2, 3, 4, 5 and 6 showed the amplified RT-PCR products at 532 bp.

Fig (1): Phylogenetic tree of nucleotide sequence of S1 fragment of three local ARV isolates showing the relationship among two Egyptian ARV isolates and other ARV strains using Clustal method of DNASTAR software package.

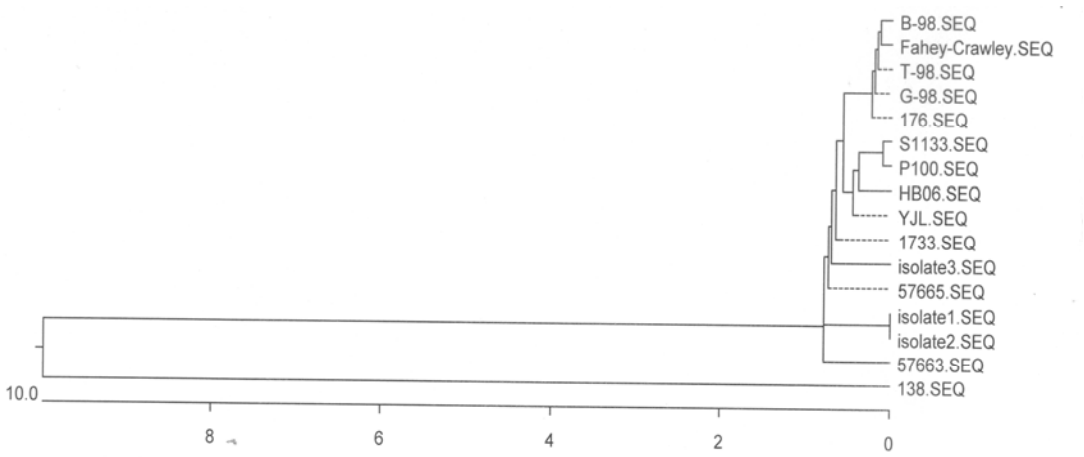
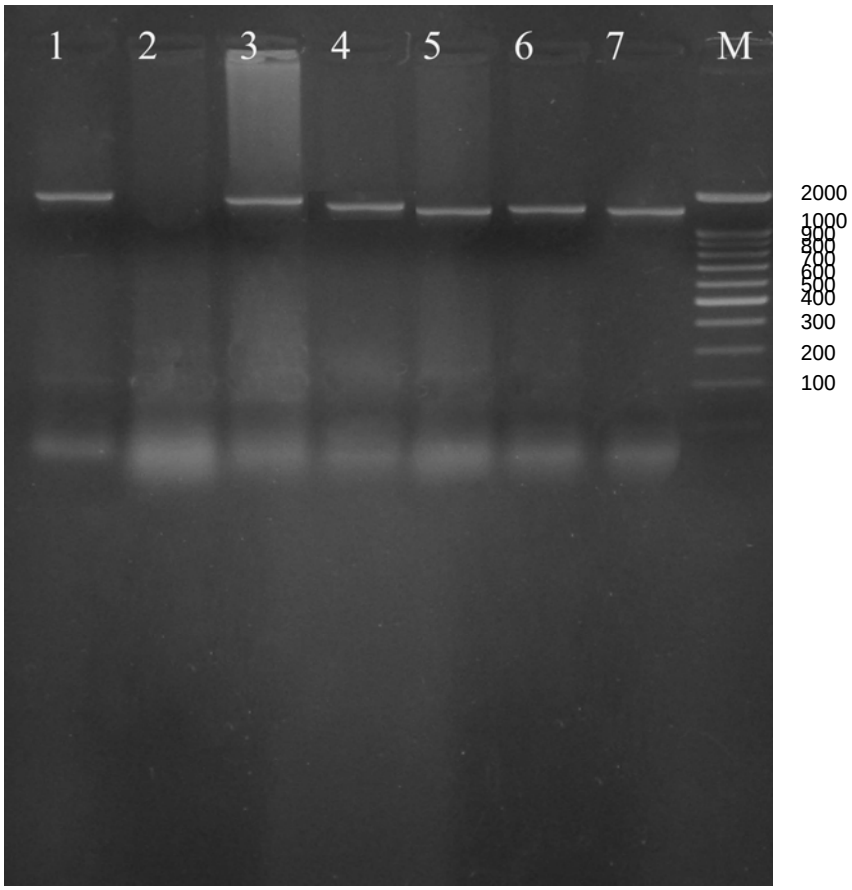


Photo (3): Electrophoretic pattern of RT-PCR assay of S1 gene of IBV



Lane M showed 2 Kb DNA ladder
Lane 1 showed amplified positive control sample.
Lane 2 showed no amplification in negative control sample.
Lanes 3, 4, 5, 6 and 7 showed the amplified RT-PCR products at 1596 bp

Fig (3): Phylogenetic tree based on nucleotide sequence of HVR of four local IBDV isolates showing the relationship among three Egyptian IBDV isolates and other IBDV strains using Clustal method of DNASTAR software package.

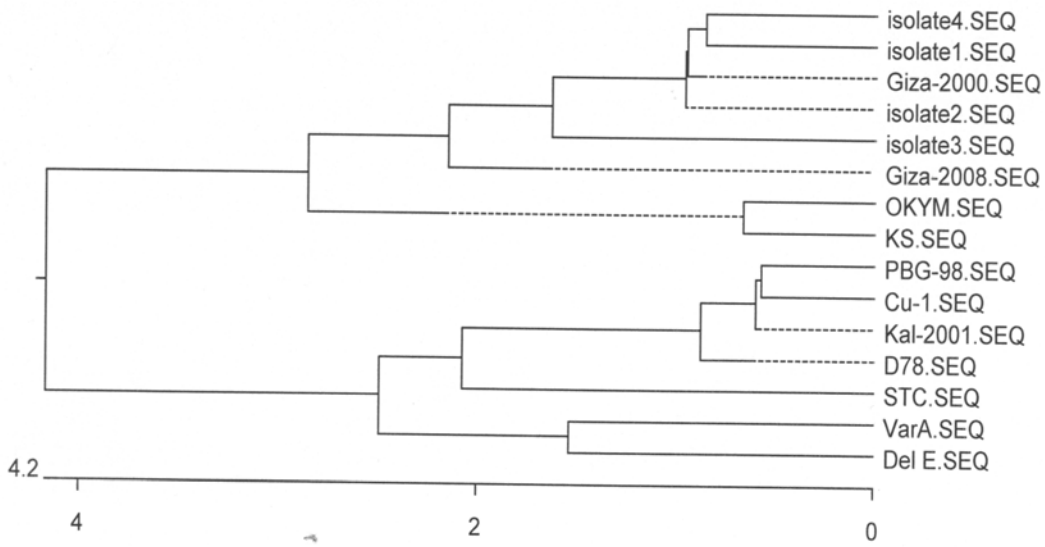
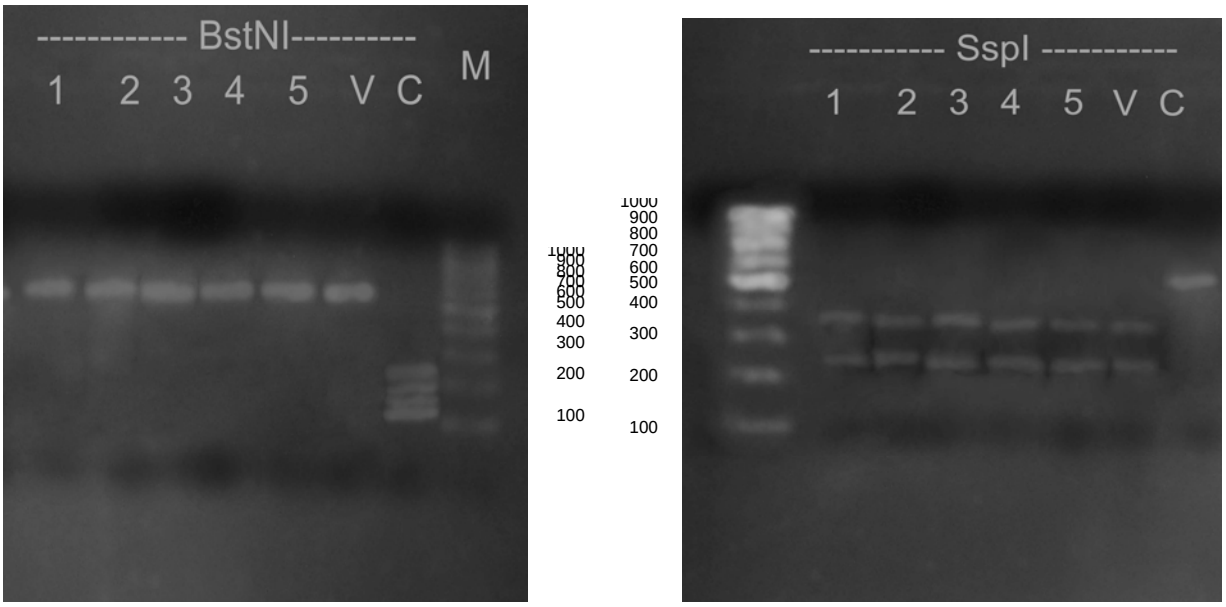


Photo (5): Electrophoretic pattern of RFLP for the hypervariable region of the VP2 gene of 5 IBDV isolates digested with BstNI (left side) and SspI (right side) restriction enzymes



Lane M showed 100 DNA ladder
Lane C showed positive control classical IBDV strain sample.
Lane V showed positive control very virulent IBDV strain sample.
Lanes 1, 2, 3, 4, and 5 (isolates code 1, 5, 8, 20 and 22) showed negative results not digested and remain as it is (643 bp) in case of BstNI restriction enzyme but positive results as it digested and yield fragments of 390 and 253 bp in case of SspI restriction enzyme.

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