Conventional and Molecular Detection of Newcastle Disease and Infectious Bursal Disease in Chickens

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ABSTRACT

The present study was undertaken to compare different diagnostic procedures for the detection of Newcastle disease and Infectious bursal disease in broilers and layers (during the period from March 2011 to February 2012 in the laboratory of the Department of Microbiology and Pathology, Faculty of Veterinary Medicine, University Putra Malaysia (UPM) .A total of 187 sick and dead chickens (63 broilers and 124 layers) of different ages (1 week to >15 weeks) were collected from 12 selective poultry farms (4 broilers and 8 layers). Clinically, 7 (14.89%) of 63 affected broiler and 27 (30.68%) of 124 affected layer chickens were diagnosed as Newcastle disease (ND) whereas, 11 (23.4%) of 63 affected broiler and 6 (4.82%) of the 124 affected layer birds were diagnosed as IBD on the basis of clinical history, clinical signs and postmortem findings. Virus isolation from field samples was performed by inoculating each suspected sample into 10-day-old chicken embryos. Out of 34 ND suspected field samples, 26 (5 broilers and 21 layers) were positive for NDV isolation and 11 (8 broilers and 3 layers) of 17 IBD suspected field samples, were positive for IBDV isolation. For confirmatory diagnosis, virus detection was confirmed by serological tests (HI and AGID) and RT-PCR assay. Out of 34 clinically diagnosed ND field samples, 20 (5 broiler and 15 layer) were positive by RT-PCR assay and 15 (10 broiler and 5 layer) of 17 IBD suspected field samples, were positive by both AGIDT and RT-PCR assay. Of the 26 HA positive NDV suspected AF, 19 (4 broilers and 15 layers) were positive by both HI and RT-PCR assay whereas, 10 (7 broilers and 3 layers) of 11 IBDV isolation positive tissue suspension were positive by both AGIDT and RT-PCR assay in the laboratory. Therefore, it may be concluded that serological (HI and AGIDT) and molecular (RT-PCR) techniques which allow rapid identification of most of samples are the reliable, sensitive, specific and more accurate methods to detect the viruses for the confirmatory diagnosis of diseases.

Key words: Clinical diagnosis, NDV, IBDV, HI, AGIDT, RT-PCR assay.

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease that affects over 250 species of birds of all age groups (Alexander, 1997) and it is caused by Newcastle disease virus (NDV) which is a linear, non-segmented single stranded, enveloped, negative sense RNA virus belonging to the genus Rubula virus of sub-family *Paramyxovirinae* and family *Paramyxoviridae* (Barbezange and Jestin, 2005). NDV differs in virulence and has been grouped into 5 pathotypes: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric (Beard and Hanson, 1984).

ND is mostly caused by velogenic strains of NDV than mesogenic or lentogenic strains. The disease produced by mesogenic strains may cause mortality that can reach 25% whereas, those by velogenic strains maybe reach up to100% and it varies from 80-90% in

the adults (Eisa and Omer, 1984 and Claudia *et al.,* 1996). The major clinical signs of ND are depression, weakness, loss of appetite, dehydration, inability to stand, cyanosis of comb and wattle, greenish watery diarrhoea, nasal and eye discharges, decreased egg production, loss of weight followed by death (Pazhanivel *et al.,* 2002). Gross lesions are petechial hemorrhages and ulcers with raised borders on the mucosa of proventriculus, pneumonic lungs, and haemorrhages in trachea, air sacs, brain and spleen (Pazhanivel *et al.,* 2002).

Infectious bursal disease (IBD) is a highly contagious acute viral disease of young chickens of 3-6 weeks old that causes a fatality or immunosuppression by damaging bursa of Fabricius and impaired growth of young chickens which results significant economic losses in the poultry industry (Lukert and Saif, 1997 and Islam *et al.,* 2005). The causal agent of IBD is

infectious bursal disease virus (IBDV), a non-enveloped double stranded RNA (dsRNA) virus belonging to the family *Birnaviridae* (Jackwood *et al.,* 1984). IBDV strains have been classified into two distinct serotypes 1, pathogenic and 2, non-pathogenic (Ismail *et al.,* 1988 and Van den Berg, 2000).

The disease is manifested by debilitate, dehydration and the development of depression with watery diarrhea, swollen and blood stained vent (Islam and Samad, 2004a). Severity of the signs depends on the virus strain and the age and breed of the chickens (Van den Berg *et al.*, 1991a). Infection with less virulent strains may not show obvious clinical signs but the birds may have fibrotic or cystic bursa of Fabricius that become atrophied prematurely (before six months of age) and may die of infections by agents that would not usually cause disease in immunocompetent birds (*The Merck Veterinary Manual*. 2006). The postmortem findings were haemorrhages in the thigh/pectoral muscles, enlarged, edematous and hyperemic bursa or atrophic in chronic cases and hemorrhage in the junction between gizzard and proventriculus (Chettele *et al.* 1989). Though gross lesions of IBD affected poultry are considered sufficient for diagnosis but is sometimes confused with other diseases (Banda, 2002).

The detection and differentiation of NDV are based on virus isolation using embryonated chicken eggs, followed by an in *vivo* determination of pathogenicity in chickens, such as the intracerebral pathogenicity index (ICPI) in 1-day-old chicks, the intravenous pathogenicity index (IVPI) in 6-week-old chickens, or the mean death time (MDT) in chicken embryos (Alexander, 1988). However, these tests are not effective and time consuming (Aldous, *et al.* 2001).

Various diagnostic methods like haemagglutination inhibition (HI) test, indirect haemagglutination (IHA) test, virus neutralization test (VNT), enzyme linked immunosorbent assay (ELISA), fluorescent antibody technique (FAT), plaque reduction neutralization test (PRNT) and agar gel immunodiffusion test (AGIDT) are used limitedly to detect NDV and IBDV. Molecular techniques like reverse transcriptase polymerase chain reaction (RT-PCR) have been frequently used all over the world to detect viruses from the field samples (Kant *et al.,* 1997; Liu *et al.,* 1998; Gohm *et al.,* 2000 and Mathivanan *et al.*, 2004).

Clinical manifestations and postmortem findings of affected birds may aid to diagnose a disease but laboratory diagnosis is necessary for confirmation of the diseases (Banda, 2002).

Identification of poultry diseases at field level is limited within recording of clinical history and signs and post-mortem findings where there is every chance of wrong diagnosis because the signs and post-mortem findings are more or less similar in most of the diseases. Therefore, the present study was undertaken to find out a relationship among the disease diagnostic parameters, i.e; clinical signs and postmortem lesions, organism isolation, serological tests and molecular methods for the diagnosis of Newcastle and infectious bursal diseases.

MATERIALS AND METHODS

Samples for the isolation of viruses

A full amount of samples 187 (63 broiler and 124 layer) sick and dead chickens aged between 1 to >15 weeks were collected from 12 (4 broiler and 8 layer) farms, which were subjected for post-mortem examination and collection of different tissue samples (trachea, lung, spleen, soft palate, colon, bursa and brain) for successful isolation and identification of viruses.

Clinical diagnosis of viral diseases

Clinical diagnosis was made on the basis of clinical history from the responsible persons of the farms, recorded clinical signs and gross lesions of affected chickens.

Preparation of hyper-immune serum

Four 6 month-old non-vaccinated chickens were vaccinated using live NDV and IBDV vaccine at day 7th, 14th and 21st. Hyper-immune serum was separated from the blood collected from vaccinated chickens and preserved at -20°C until further use.

Isolation of NDV and IBDV in avian embryo

The specific pathogen free (SPF) 10 day-old embryonated chicken eggs were inoculated through allantoic cavity route for NDV and chorio-allantoic membrane (CAM) route for IBDV at 0.2 ml (0.1 ml virus suspension + 0.1 ml antibiotic mixture) of inoculums. Allantoic fluid was collected and tested by slide HA test. Those manifesting HA-positive (clumping of RBC) were collected, as a source of ND virus. For IBDV samples of CAM were collected with PBS (phosphate buffered saline) to prepare 50% suspension and stored at -80°C for further use.

Serological Methods

Hemagglutination Inhibition (HI) test: HA positive samples (AF) were subjected for HI test. A 25 μl of PBS was taken to all wells of each numbered column and 25 μl of anti-NDV hyper-immune serum was added to the every first well. Serial two fold dilution of sera was made and 25 μl of control antigen was added to the well and test antigens were taken to the remaining wells. A volume of 25 μl of PBS was added to serum control plate instead of antigen and kept for 60 min at room temperature. Then 50 μl of 0.6% cRBC was added to all wells and kept at room temperature at least for 25 min to record the result (Anon, 1971).

Agar gel immunodiffusion test (AGIDT): All the collected field samples positive (50% inocula) and tissue suspension (CAM and embryo) were used for AGIDT (Wood *et al*. 1979). Briefly, the central well of a glass slide coated with melted agarose gel was loaded with known hyperimmune sera against IBDV and peripheral wells with reference antigen of IBDVs and bursal suspensions. Slides were kept in moist chamber

for 48-72 hours at 40C and observed for antigen antibody reaction in the form of appearance of precipitation lines in between the central and peripheral wells.

Molecular method

Extraction of viral RNA: Viral RNA of the viruses was extracted from 140 μl of IBDV and NDV suspected field samples (inocula), laboratory samples (CAM and embryo suspension for IBDV and AF for NDV) using QIAamp viral RNA mini kit (QIAGEN,

Hilden, Germany) according to the manufacturer's procedure. The RNA was extracted in 60 μl of elution buffer and used as template directly for RTPCR assay or stored at -80ºC until further use.

Selection of oligonucleotide primers: Newcastle disease virus specific sense and complementary primer sets and infectious bursal disease virus type specific sense and complementary primers designed by using OLIGO 2 software were used for RT-PCR.

Table 1. Primers used for RT-PCR of IBDV and NDV

Primers	Primers Sequence (5´-3´)	Nucleotide Position
Vvfp 775 (Sense)	5´-AATTCTCATCACAGTACCAAG -3´	253 bp
Vvrp 1028 (Antisense)	5´-GCTGGTTGGAATCACAAT -3´	
NDV/Fa (Sense)	5'TCAACATATACACCTCATCCCAGACAGG-3'	387 bp
NDV/Ra (Antisense)	5'-CTGCCACTGCTAGTTGGGATAATCC-3'	

RT-PCR for the detection of ND and IBD viral genome

Synthesis of cDNA from RNA

A volume of 4 μl of eluted RNA of NDV or IBDV and 8.3μl dH2O were mixed properly and allowed for linearization of coiled RNA in thermocycler (BIORAD®, USA) maintaining 94°C temperatures for 5 min followed by 2 min snap cooling on ice to stay liberalized. Meanwhile, for the synthesis of cDNA from RNA of NDV or IBDV, reaction mixture 1 containing 5XRT buffer 4.0 μl, 10 mM dNTP 2.0 μl, prime RNase inhibitor 1.0 μl, AMV-RT 0.2 μl, primer (RH 100 pmol) 0.5 μl, Template RNA 4 μl, dH2O 8.3μl were prepared and kept on ice. After adding this reaction mixture on to the PCR tube containing linearized RNA of NDV or IBDV placed into the thermocycler and followed the thermal profile as 42°C for 40 min followed by 85°C for 5 min.

Synthesis of DNA from cDNA of NDV

For the synthesis of DNA from cDNA of NDV, reaction mixture-2 were used as 50 μl volume containing 10X LA buffer 5.0 μl, 25 mM MgCl2 2.0 μl, 10 mM dNTP 2.0 μl, LA-Taq 0.2 μl, NDV/Fa primer 0.8 μl and NDV/Ra primer 0.8 μl, cDNA 1.5 μl, and DEPC 37.7 μl to each tube and mixed with minispin. The tubes were immediately placed to the thermocycler and maintained the thermal profile at 94°C for 2 min, 30 cycles were continued at 94°C for 30 seconds for denaturation, 45°C for 45 seconds for annealing, 60°C for 1 min for elongation and final elongation at 60°C for 10 min.

Synthesis of DNA from cDNA of IBDV

For the synthesis of DNA from cDNA of NDV, reaction mixture-2 were used as 50 μl volume containing 10X LA buffer 5.0 μl, 25 mM MgCl2 2.0 μl, 10 mM dNTP 2.0 μl, LA-Taq 0.2 μl, Vvfp 775 primer 0.8 μl and Vvrp 1028 primer 0.8 μl (for IBDV), cDNA 1.5 μl, and DEPC 37.7 μl to each tube and mixed with minispin. The tubes were immediately placed to the thermocycler and maintained the thermal profile at 94°C for 2 min, 30 cycles were continued at 94°C for

30 seconds for denaturation, 45°C for 45 seconds for annealing, 60°C for 1 min for elongation and final elongation at 60°C for 10 min.

RESULTS

Clinical diagnosis of diseases

Newcastle disease (ND): For clinical sings, 7 (14.89%) birds of the 63 affected broiler chickens and 27 (30.68%) of the 124 affected layer chickens were dedicated as ND (Table 1). The most familiar clinical signs were edema of the head-face-wattles, twisted neck and paralysis (Figure 1), greenish diarrhea, cessation of egg production, soft-shelled egg and death which were similar with the findings of Beach (1942), Banerjee *et al.* (1994) and Alexander (1997).

Most commonly observed postmortem lesions were pin point hemorrhages at the tip of proventricular glands (Figur 3- 4), hemorrhagic ulcers in intestinal wall and caecal tonsils, petechial hemorrhage in colon, hemorrhagic lungs, tracheitis (Figure 2) with congestion and catarrhal exudates. These findings agree with the findings of Kotani *et al.* (1987), Crespo *et al.* (1999), Talha *et al.* (1999) and Pazhanivel *et al.* (2002).

Infectious bursal disease (IBD): A total of 11 (23.4%) of the 63 affected broiler and 6 (4.82%) of the 124 affected layer birds were diagnosed as IBD (Table 1) and observed clinical signs were high mortality, unsteady gait, ruffled feathers (Figure 5), urate containing diarrhea and sudden death which correspond with the findings of Lukert and Saif (2003), Islam and Samad (2004).

The postmortem findings were hemorrhages in the thigh/pectoral muscles (Figure 6), enlarged, edematous and hyperemic bursa (Figure 7) with bloody or mucoid contents (Figure 8) or atrophic in chronic cases and hemorrhage in the junction between gizzard and proventriculus which support the findings of Chettele *et al.* (1989), Lukert and Hitchner (1984) and Islam and Samad (2004).

Table 1. Clinical diagnosis of Newcastle and infectious bursal disease suspected cases

Name of the diseases	Total number of study samples		Clinically diagnosed			Prevalence (%)		
	Broiler	Layer	Broiler	Layer	Total	Broiler	Layer	Overall
Newcastle disease			7	27	34	14.89	30.68	53.96
Infectious bursal disease	63	124	11	6	17	23.4	4.82	17.7

Clinical and laboratory diagnoses of Newcastle and infectious Bursal diseases of chickens

Figure 1. NDV infected layer chicken of 29 weeks of age showing nervous signs (twisted neck and paralysis)

Figure 2. Chicken infected with NDV showing hemorrhages in the trachea chicken infected with

Figure 3 and 4. Presence of ulcer and pin point haemorrhages in the proventriculus of chickens affected with ND
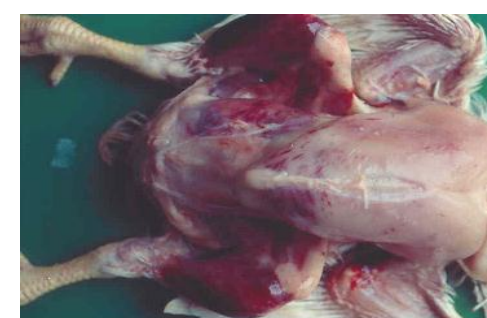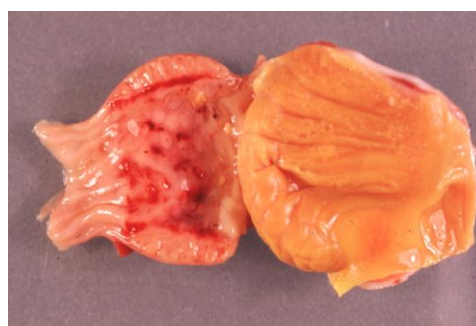

Figure 5. Broiler chicken of 20 days infected with IBDV showing depression and unsteady

Figure 6. Chicken affected with IBD showing hemorrhages in the thigh muscles

Figure 5. Swollen and hyperemic bursa of Fabricius

Figure 6. Cut section of bursa of IBDV infected

of IBD affected broiler chicken

Laboratory diagnosis

Isolation of NDV in chicken embryos: A total of 26 (76.47%) samples from 34 clinically diagnosed ND affected chickens, were positive for virus isolation in embryonated eggs (Table 2). In all positive cases embryos died within 24 to 96 hours of post inoculation (Spackman et al., 2003 and Woolock, 2008).

All of the 26 AF samples showed positive rapid slide HA activity within few seconds which indicated that the isolates were hemagglutinating viruses (Okoye, 1983). The HA positive samples were subjected for the determination of virus titer which were within a range of 64- 512.

broiler chickens showing bloody and mucoid contents

Isolation of IBDV in chicken embryos: Out of the 17 clinically diagnosed IBD affected samples, 11 (64%) were positive for isolation of virus which were negative to rapid slide HA test (Table 2). In positive cases the embryos were died within 24 to 96 hours of post-inoculation. The CAM was thickened, dead embryos were congested and hemorrhagic (petechial and ecchymotic) along the feather tracts, toe and cerebral area which were similar to the findings of Hitchner (1970) and Takase et al. (1996). The reduced rate of virus isolation may be due to absence or low concentration of virus in the remaining six inoculums or due to the presence of maternal antibody in the embryonated eggs (Rosales et al., 1989).

Table 2. Results of virus isolation using embryonated chicken embryos

Name of the Diseases	Samples inoculated			Virus isolation positive			
	Broiler	Layer	Total	Broiler	Layer	Total	
						No.	%
ND	7	27	34	5	21	26	67.47%
IBD	11	6	17	8	3	11	64%

Hemagglutination inhibition test using hyper-immune serum against NDV

Out of the 26 HA positive AF, 19 (73.08%) were neutralized by anti-NDV hyper-immune serum whereas, 7 were not neutralized which indicated these were other than NDV (Table 3). The result of this study partially agrees with the findings of Alders et al. (1994), Manin et al., (2002) and Singh et al. (2005) who successfully detected and differentiated NDV from the field samples by HI test using NDV specific polyclonal serum.

AGID test using hyper-immune serum against IBDV

Prominent white line of precipitation was noticed between known positive anti-IBDV hyper-immune serum of the central well and bursal homogenates of the peripheral wells due to antigen and antibody reaction within 24- 48 hr. By AGIDT, out of 17 field samples, 15 (10 broiler and 5 layer) samples and of the 11 laboratory samples, 10 (7 broiler and 3 layer) samples were positive for IBDV (Table 3). The results are in agreement with the findings of Karunakaran et al. (1993), Muhammad et al. (1996), Gupta et al. (2001).

No line of precipitation was observed in 2 (8.57%) field samples and one tissue suspension that were considered as negative for IBDV antigen.

RT-PCR for Newcastle disease virus (NDV)

The nucleic acid based detection tests like RT-PCR have been used for the detection of viruses (Liu et al., 1994 and Kataria et al., 2000). Viral RNA was extracted from both 34 field samples and 26 HA positive AF for the detection of NDV genome by RT-PCR using NDV specific primers. Out of 34 field samples, 26 (5 broilers and 21 layers) samples and of the 26 (5 broilers and 21 layers) AF, 19 (4 broilers and 15 layers) samples were positive for the detection of NDV viral genome (Table 3). This low rate of detection may be due to presence of hemagglutinating viruses other than NDV in the AF. A clear and distinct band of RT-PCR product was appeared at the position of 387 bp with the standard 100 bp DNA ladder passed through 2% Agarose gel electrophoresis (Figure 9). The results almost similar with the findings of Kant et al. (1997), Nanthakumar et al. (2000), Gohm et al. (2000) and Singh et al. (2005).
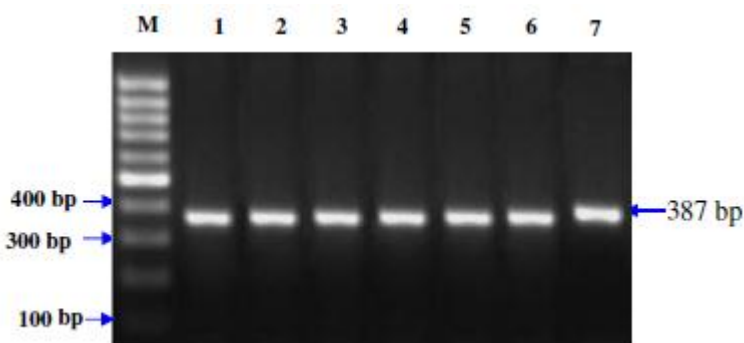

Figure 9. RT-PCR products of NDV (387 bp) from field samples showing specific bands on 2% agarose gel electrophoresis. M = 100 bp DNA Marker, Lane 1= reference NDV and Lane 2-7 = field samples
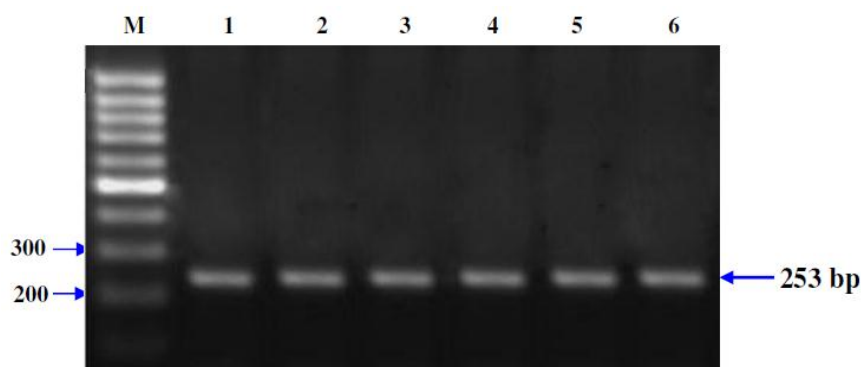
Figure 10. RT-PCR products (253 bp) of IBDV from allantoic fluids analyzed using 2% agarose gel electrophoresis. M = 100 bp DNA Marker, Lane 1= reference IBDV and Lane 2-6 = field samples of IBD

Table 3. Serological* and molecular diagnosis (RT-PCR) of Newcastle disease and infectious bursal disease

		Serological test						Molecular test					
		Broiler		Layer		Total		Broiler		Layer		Total	
		Study sample	+ve	Study sample	+ve	Study sample	+ve	Study sample	+ve	Study sample	+ve	Study sample	+ve
ND	Field samples	-	-	-	-	-	-	7	5	27	15	34	20
	AF	5	4	21	15	26	19	5	4	21	15	26	19
IBD	Field samples	11	10	6	5	17	15	11	10	6	5	17	15
	CAM + embryo suspension	8	7	3	3	11	10	8	7	3	3	11	10

*Agar gel immunodiffusion test for IBDV and Hemagglutination Inhibition test for NDV

Comparison between the clinical and laboratory diagnoses of viral diseases

A total of 187 dead and sick birds were examined under this study, of which 34 birds were clinically diagnosed as Newcastle disease and 15 as infectious bursal diseases. All the clinically diagnosed samples were inoculated in avian embryos that results 20 virus isolation positive for NDV and 11 for IBDV. A total of 19 samples out of 26 NDV isolates were neutralized by anti-NDV hyper-immune serum whereas, AGIDT detected 15 of 17 field samples and 10 of 11 laboratory isolates as IBDV. Finally all the samples were subjected for

molecular detection method using RT-PCR. Out of 34 field samples, 26 and of the 26 NDV isolates, 19 were positive for the detection of ND viral genome by RT-PCR. In case of IBDV, 15 of 17 field samples and 10 of 11 laboratory isolates were positive by RT-PCR (Table 4).

In this study, the result revealed that all the clinically diagnosed ND and IBD samples were not positive by laboratory diagnostic method (serological and molecular method) which may be other than ND or IBD. On the other hand both the serological using virus specific hyperimmune serum and molecular methods using viral genome specific primers diagnosed positively similar number of field and laboratory samples in both cases of ND and IBD which indicated higher accuracy of disease diagnosis (Lee et al., 1994; Kianizadeh, et al., 1999 and Singh, et al., 2005).

Table 4. Comparison between the clinical and laboratory diagnosis of Newcastle and Infectious Bursal diseases

			Serological detection**						Molecular detection(RTPCR)					
			Field samples			Laboratory samples*			Field samples			Laboratory samples*		
			SS	+ve	%	SS	+ve	%	SS	+ve	%	SS	+ve	%
ND	34	26	-	-	-	26	19	73.03	34	20	58.82	26	19	73.03
IBD	17	11	17	15	88.23	11	10	90.90	17	15	88.23	11	10	90.90

Serological detection**= Agar gel immunodiffusion test for IBDV and Hemagglutination Inhibition test for NDV, Laboratory samples*= Virus isolation positive allantoic fluid for ND and CAM+embryo suspension for IBDV, SS= Study samples, VI= Virus isolation.

Therefore, it may be concluded that serological (HI and AGIDT) and molecular (RT-PCR) techniques which allow rapid identification of most of samples are the reliable, sensitive, specific and more accurate

methods to detect the viruses for the confirmatory diagnosis of diseases.

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