Pak. J. Agri. Sci., Vol. 51(3), 703-709; 2014
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

COMPARATIVE EVALUATION OF CONVENTIONAL AND MOLECULAR DIAGNOSTIC METHODS FOR NEWCASTLE DISEASE DURING AN OUTBREAK IN PUNJAB, PAKISTAN

Mohsan Javed[1,2*], Farzana Rizvi[2], Abdul Whab Manzoor[3], Mian Muhammad Awais[4], Rai Shafqat Ali Khan[1] and Abrar ul Haq Khan[5]

[1]Poultry Research Institute, Rawalpindi, Pakistan; [2]Depatment of Pathology, University of Agriculture, Faisalabad, Pakistan; [3]Veterinary Research Institute, Lahore, Pakistan; [4]Department of Pathobiology, Subcampus Jhang, University of Veterinary and Animal Sciences, Lahore, Pakistan; [5]Institute of Microbiology, University of Agriculture Faisalabad, Pakistan.
[*]Corresponding author's e-mail: rmjj84@gmail.com

Field survey of broiler poultry farms suspected to be infected with Newcastle disease (ND) was carried out during an outbreak in district Faisalabad, Punjab in 2012. The validity of different diagnostic methods; like virus isolation, identification and two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), was assessed and compared for the effective and rapid diagnosis of ND virus (NDV). Moreover, suitability of different postmortem samples (pooled tissues) and clinical samples (tracheal swabs) was also evaluated for efficient diagnosis. A higher rate of NDV detection was found with pooled tissue samples than tracheal swabs with virus isolation, identification and RT-PCR tests. Coefficient of agreement (K value) was calculated and a perfect and substantial agreement was found between both antigen-detection assays with K value of 0.82 and 0.72 when applied on pooled tissues samples and tracheal swabs, respectively. After molecular detection of NDV, a significant relationship was observed between nervous signs and RT-PCR ($P<1.00$), while a non significant relationship of RT-PCR results was recorded with enteric and respiratory signs ($P<0.08$- 0.48). Data obtained from the field study suggests that pooled tissue samples are more suitable for the detection of NDV than tracheal swabs with both of the diagnostic techniques. Moreover, NDV detection rate was higher with RT-PCR than the conventional virus isolation and identification method along with an additional advantage of being quick to perform which is a basic necessity in case of ND outbreaks.
Keywords: ND virus, RT-PCR, histopathology, virus isolation

INTRODUCTION

Newcastle disease (ND), defined as a list A disease by the Office International des Epizooties (OIE), is a highly contagious avian disease, which is worldwide and causes severe economical losses (OIE, 2012). Several outbreaks of ND have occurred in commercial as well as backyard poultry all over the world. The first outbreak of ND was occurred in 1926 in Java, Indonesia and in Newcastle-upon-Tyne England in 1927. After that 239 outbreaks occurred in European Union countries in1994 and 254 outbreaks in Italy in 2000 (Alexander *et al*., 2004). Furthermore two outbreaks of virulent ND were reported in Australia in 1999 and 2000 (Kirkland, 2000; Westbury, 2001). A recent outbreak of ND occurred in Punjab, Pakistan in 2012 and caused an economical loss of about 6 billion PKR during a period of 5 months (February to June 2012) and about 44.59 million broiler chickens were perished by this infection (Anonymous, 2012).

Newcastle disease is caused by ND virus (NDV) which is designated as avian paramyxovirus serotype 1 (APMV 1) (Alexander, 1997) and belongs to the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus*. (Mayo, 2002). About the 236 species of both pet as well as free living birds can be infected with ND, in addition to domestic avian species (chicken, turkey, goose, duck and pigeon) (Kaleta and Baldauf, 1988). Among poultry, chickens are most susceptible, whereas ducks and geese are relatively resistant. The Newcastle disease virus is enveloped and has a negative-sense, single-stranded RNA genome of approximately 15kb (de Leeuw and Peeters, 1999) which codes for six proteins, including an RNA-directed RNA polymerase (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P) and nucleoprotein (N) (Lamb and Kolakofsky, 1996).

Appropriate diagnosis of ND plays an important role in the early detection of the infection as well as to control the outbreaks. Various serological tests like Haemagglutination (HA) and Haemagglutination Inhibition (HI) test (MAFF, 1984), Enzyme linked immunosorbent assay (ELISA) (Roy and Venugopalan, 1999), Fluorescent Antibody Technique

(FAT) (Wu *et al*., 1999), *In-Situ* Hybridization (ISH) (Oldoni*et al*., 2005) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (Jestin *et al*., 1991; Ghom *et al*., 2000) with several modifications like real time (rt) PCR (Wise *et al*., 2004) and multiplex nested RT-PCR (Nguyen *et al*., 2012) are used for the diagnosis as well as to study the pathogenesis of NDV. But the most widely used conventional method is the isolation of virus in embryonated chicken eggs followed by HI assay with the use of specific hyper immune serum raised against NDV (OIE, 2012). The above said method is labor intensive and time consuming (Aldous *et al.,* 2001) as the sample processing time for this method is not compatible with high demand of poultry industry during an outbreak. So in case of outbreak and emergency situations the selection of proper samples and best diagnostic technique has a fundamental importance for early and accurate detection of infection as well as in controlling outbreak.

In this report, the clinico-pathological study has been demonstrated in naturally infected broilers during recent outbreak to study the clinical signs, gross and histological alterations in various tissues. Moreover the samples collected during the epidemic were used to assess the validity of conventional and molecular diagnostic methods as well as the suitability of different samples was also evaluated for rapid and accurate detection of NDV during the outbreak.

MATERIALS AND METHODS

***Sample collection*:** A field survey of commercial broiler poultry farms (n=50) suspected of being infected with ND was carried out in district Faisalabad, Pakistan during an epidemic of ND in March to April 2012. The suspected birds either live or fresh mortality (n=10) from each suspected infected premises were brought to the laboratory. Clinical signs and gross lesions in the diseased birds of each farm were observed and recorded to compare with the finding of RT-PCR. Clinical samples (tracheal swabs) were collected from each bird with sterile dry cotton swabs, and visceral organs (lungs, trachea, kidney, spleen, intestine, liver and proventriculus) were collected from the same dead or sacrificed birds for histopathology, virus isolation and direct RT-PCR. Tracheal swabs and tissues collected from each farm were pooled (10 swabs/organs per pool) separately and stored at -20 ^{o}C till further use.

***Histopathological studies*:** The visceral organs including intestine, proventriculus and liver of suspected birds were subjected to histopathology as described by Bancroft and Gamble (2007).

***Virus isolation and RT-PCR*:** The pooled visceral organs including lung, trachea, spleen, kidney collected from each suspected farm were diluted separately with normal saline (10% w/v) and homogenized in tissue homogenizer. Homogenate thus obtained was subjected to centrifugation (1000×*g* for 10 minutes at 4°C), supernatant was separated and antibiotics (penicillin @ 2000 units/ml, streptomycin @ 2 mg/ml, gentamycin @ 50 µg/mg and nystatin @ 1000 units/ml) were added to the homogenate to avoid the bacterial and fungal contamination (OIE, 2012). Then, it was used for virus isolation and direct RNA extraction for RT-PCR. Tracheal swabs suspended in Phosphate Buffered Saline (PBS) were added with antibiotics 1ml/swab and incubated at room temperature for 1 hr. After centrifugation supernatant was collected and subjected to virus isolation and Direct RNA extraction for RT-PCR (OIE, 2012)

***(a) Virus isolation and identification*:** Virus was isolated from both types of the sample by their inoculation in 9-day-old embryonated chicken eggs obtained from Poultry Research Farm of the Institute, according to methodology described by OIE (2012). Serological identification of NDV was performed by using the HI assay according to the methodology described by Alexander *et al*. (1998). Anti NDV hyperimmune serum was raised in chickens and used in HI assay (Iqbal *et al*., 2003).

***(b) Reverse transcriptase polymerase chain reaction*:** Viral RNA was extracted separately from 200µl of both of the tissue homogenate and tracheal swab suspension by using the GF-1 viral nucleic acid extraction kit (GF-RD-100, Vivantis) as recommended by the manufacturer. cDNA was synthesized by using the RevertAid^{TM} First Strand cDNA Synthesis kit (Fermentas). Briefly, two mixtures were prepared separately. Mixture-I was composed of 5µl of RNA, 2µl of random hexamer primer (0.2µg/µl) and 3µl of DEPC treated water, while the mixture-II was prepared by adding the 5µl of 5x RT buffer, 1µl of bovine serum albumin (1mg/ml), 1µl of 10mM dNTPs mix, 1µl M-MuLV reverse transcriptase (250 units), 2µl 50mM DDT, 1µl ribonuclease inhibitor 50 units (Vivantis). Mixture-I and II were mixed and incubated at 37ºC for 1 hr. Reaction was stopped by heating at 70ºC for 10 min to inactivate the reverse transciptase enzyme and cDNA was stored at -20ºC till further use. A set of oligonucleotide primers according to Creelan *et al.* (2002) was used in the study. The sequence of primers were as follows: NDV/sense primer (5’ - GGT GAG TCT ATC CGG ARG ATA CAA G – 3’) and NDV/antisense primer (5’ - TCA TTG GTT GCR GCA ATG CTC T– 3’). A 50 bp DNA ladder was run and vaccine strain LaSota (Nobilis®, USA) was included as positive control. The expected size of PCR product was 202 bp.

The PCR was carried out by adding 2µl of cDNA, 2µl of 10X PCR buffer, 0.5µl dNTP, 1.4µl of $MgCl_2$ (50mM) and 0.5µl Taq polymerase (Fermentas). Thermocycler (Peq Lab Primus 25 advanced USA) conditions were: 2 min at 94ºC (initial denaturation), followed by 40 cycles of 15 s at 94ºC (denaturation), 30 s at 48ºC (annealing), 30 s at 72ºC (elongation). The PCR ended with a final elongation for 7

min at 72ºC. Amplicons were visualized on 2% agarose gel (Simethkana *et al*., 2006).

RESULTS

***Clinical signs*:** Clinical signs were observed and recorded and mainly categorized into three types; respiratory, enteric and nervous. The commonly observed clinical signs were enteric while those observed least were nervous signs. Respiratory signs were observed at 31 (62%) farms and mainly included the labored breathing, oculo- nasal discharge, while enteric signs were observed at 35 (70%) farms including greenish-mucoid and bloody diarrhea. Nervous signs were observed at 16 (32%) farms including torticollis, ophisthotonos, leg and neck paralysis. Conjunctivitis was also observed as general clinical finding at 33 (66%) infected premises Table 1.

Table 1. Prevalence of different clinical signs recorded at Newcastle disease infected broiler farms

Clinical signs	Positive farms / Total farms examined	Prevalence (%)
Conjunctivitis	33/50	66
Respiratory signs	31/50	62
Oculo-nasal Discharge	24/50	48
Laboured breathing	27/50	54
Sneezing and coughing	21/50	42
Soft swelling of face	14/50	28
Gasping	23/50	46
Enteric signs	35/50	70
Greeninsh mucoid Diarrhea	31/50	62
Bloody diarrhoea	04/50	08
Nervous signs	16/50	32
Torticollis	15/50	30
Ophisthotonos	13/50	26
Wing and leg Paralysis	15/50	30

Gross lesions: Gross lesions confined to digestive system were observed at 33 (66%) farms including the petechial, small ecchymotic hemorrhages and necrotic plaques diffused in the mucosa of the proventriculus Fig.1(A). Prominent ulceration and deep sloughing of mucosa was observed in intestine Fig.1(C). Necrosed and haemorrhagic ceacal tonsils were observed at 29 (58%) farms visited. Regarding the respiratory tract; tracheal haemorrhages, congested, edematous lungs and opaque air sacs containing exudate were found as major gross alterations and observed at 31 (62%) farms. Marked spleenomegaly, disseminated focal necrosis and mottling were observed at 19 (38%) farms. Regarding the nervous system no significant gross lesions were observed during the survey.

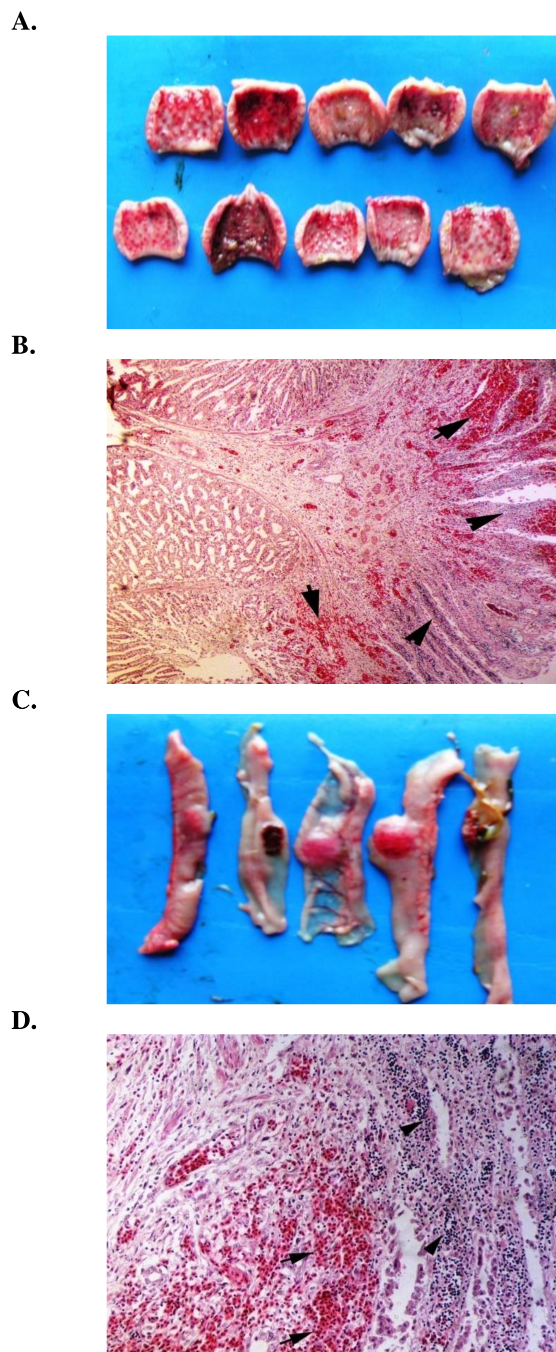


Figure 1. Petechial hemorrhages in proventriculus in natural ND infection (A). Congestion (arrows) and cellular infiltration (arrow head) in the secondary and tertiary duct area of proventriculus (B). Severe ulceration in small intestine of broilers naturally infected with ND (C). Severe hemorrhages (arrows) and excessive cellular infiltration (arrow head) in the intestine (D).

***Histopathological lesions*:** Excessive cellular infiltration was seen in intestinal villi along with severe hemorrhages and congestion in the mucosa of intestine. Necrotic changes and congestion in the inner circular layer of muscles was observed. Cellular infiltration, severe hemorrhages and congestion were seen in the secondary and tertiary duct areas of proventriculus Fig.1(B). Congestion, hemorrhages and dilated sinusoidal spaces were seen in the liver parenchyma and in-a-few tissues billiary hyperplasia was prominent. Pyknosis, karyolysis and mild degree of vacuolation were also seen. Severe hemorrhages and excessive cellular infiltration were also observed in the intestine Fig.1(D).

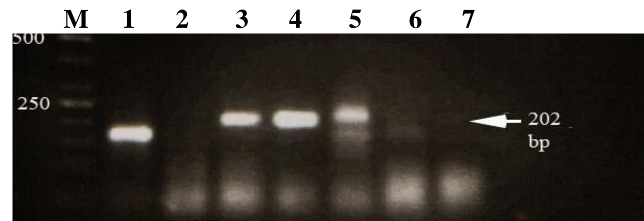
***Comparison between clinical diagnosis and RT-PCR*:** All the 50 samples were diagnosed through RT-PCR using primer set of F gene region producing a band of 202 bp size. Comparative study between the diagnostic outcome of RT-PCR and clinical findings was carried out and analyzed statistically by Chi-square test. The nervous signs were observed at 16(32%) and all of these samples were found positive through RT-PCR. Respiratory signs and gross lesions were observed at 31(62%) farms and out of these 19(38%) were detected as positive through RT-PCR. Clinical findings regarding the enteric system were observed at 35(70%) farms, of which 22(44%) samples were found positive in molecular diagnosis. Nervous signs alone or in combination with respiratory and enteric signs were significantly related with RT-PCR (P<1.00) while a non-significant relationship of enteric and respiratory signs was found with molecular diagnosis (P<0.08- 0.48) (Table 2).

Table 2. Comparison between clinical and RT-PCR based diagnosis of Newcastle disease

Clinical signs and gross lesions	Positive samples in clinical diagnosis (n=50)	Positive samples in RT-PCR (n=35)	Chi square value
N	8	8	0.000 (P<1.00)
E	8	5	0.692 (P<0.41)
R	5	3	0.500 (P<0.48)
N+E	3	3	0.000 (P<1.00)
N+R	2	2	0.000 (P<1.00)
R+E	21	11	3.125 (P<0.08)
N+R+E	3	3	0.000 (P<1.00)

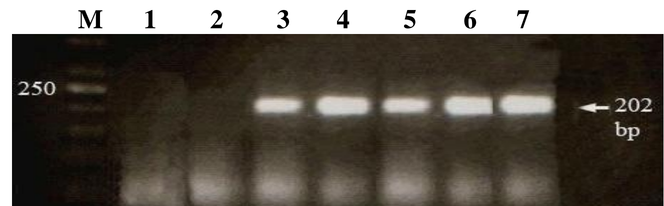
N= Nervous signs, E= Enteric signs, R= Respiratory signs, RT-PCR= Reverse Transcriptase Polymerase chain Reaction

***Comparison between virus isolation, identification and RT-PCR*:** In case of pooled tissue samples out of total 50 pooled samples 31 (62%) were detected as positive for NDV through VI while the remaining 19 (38%) samples were found negative. Both of the VI-positive and negative samples were retested through RT-PCR and all of the 31 (62%) VI-positive samples were also detected as positive while of 19 VI-negative samples 04 (8%) samples were detected as positive and remaining 15 (30%) samples were found negative. In case of tracheal swabs, out of total 50 pooled samples 23 (46%) were detected as positive in VI and remaining 27(54%) as negative samples. Again both of VI-positive and negative samples were retested through molecular diagnosis and out of 23 VI-positive samples, 21 (42%) were detected as positive, while out of total 27 VI-negative samples, 5 (10%) were detected as positive and 22(44%) were found negative. Overall assessment, in case of pooled tissue organs out of total 50 samples 08 (16%) and 09 (18%) more samples were detected as positive in VI and RT-PCR, respectively than the tracheal swab samples (Fig. 2 and 3). Coefficient of agreement (K value) was also calculated and a substantial agreement was found between the two virus/RNA detection assays with a K value of 0.82 and 0.72 in case of pooled tissue samples and tracheal swabs, respectively (Table 3).



M = DNA Ladder 50bp (Vivantis), Lane 1=positive control NDV LaSota strain (Nobilis), Lane 3, 4 and 5 = NDV +ve (pooled tissue organs), Lane 2 and 6 = NDV -ve, Lane 7= -ve control (water).

Figure 2. Electrophoretic profile of RT-PCR products showing bands of Newcastle disease virus



M= DNA Ladder (50bp) (Vivantis), Lane 3, 4, 5, 6 =NDV +ve (tracheal swabs), Lane 2= NDV –ve, Lane 7= +ve control NDV strain LaSota (Nobilis), Lane 1= - ve control (water).

Figure 3. Electrophoretic profile of RT- PCR products showing bands of Newcastle disease virus from tracheal swabs.

DISCUSSION

Newcastle disease causes one of the most serious diseases in commercial as well as backyard poultry. In the present study we compared the conventional and molecular diagnostic tools for Newcastle disease diagnosis. Moreover the suitability of different sample type was also determined.

Table 3. Comparison between virus isolation, identification and RT-PCR for detection of Newcastle disease virus using pooled tissue samples and tracheal swabs

	Pooled tissue samples			
	VI (+ve)	**VI (-ve)**	**Total two step RT-PCR sample**	**Agreement (K value)**
Two step RT-PCR (+ve)	31 (62%)	4 (8%)	35 (70%)	
Two step RT-PCR (–ve)	0	15 (30%)	15 (30%)	
Total VI samples	31 (62%)	19 (38%)	**50(100%)**	**0.82**
	Tracheal swabs			
Two step RT-PCR (+ve)	21 (42%)	05 (10%)	26 (52%)	
Two step RT-PCR (-ve)	02 (4%)	22 (44%)	24 (48%)	
Total VI Samples	23 (46%)	27 (54%)	**50 (100%)**	**0.72**

VI= Virus Isolation, Identification. RT-PCR= Reverse Transcriptase Polymerase Chain Reaction

Clinical signs observed during the study were in agreement with the findings of Brown *et al*. (1999), Kommers *et al*. (2003) and Stevens *et al*. (1976) during the natural and experimental ND infections. Gross lesions were mainly confined to the digestive tract as stated above. Similar findings have been reported by Wakamatsu *et al*. (2006) and Susta *et al*. (2010) during experimental and natural ND infections. Despite the neurotropism of virus no prominent gross lesions were present in the central nervous tissue and this finding directly supports the finding of Brown *et al*. (1999). Histopathological study of proventriculus, intestine and liver was conducted because the gross lesions were mainly confined to these organs. The histological findings have a close agreement with the findings of Alexander (2003) and Wakamatsu *et al* (2006). During VI and RT-PCR, out of total 50 pooled tissue samples 08 (16%) and 09 (18%) more samples were detected as NDV positive in VI and RT-PCR, respectively than the tracheal swabs. This could be due to variability in the predilection site of virus and the chances of viral detection decreases while testing a single organ like tracheal swab as source of NDV. The higher detection rate of NDV with pooled tissue samples than tracheal swabs correlates with the finding of Ghom *et al*. (2000) and Simethkana *et al*. (2006). Moreover K value of 0.82 and 0.72 in case of pooled tissue samples and tracheal swabs, respectively suggests a perfect and substantial agreement between VI and RT-PCR. On individual sample basis, NDV detection rate was higher with RT-PCR than virus isolation with both types of the samples, and this might be due to two main facts: (i) in VI a long time processing of sample is required as propagation of virus in embryonated chicken eggs than direct RT-PCR and during this process inactivation of the virus may occur and results into a false negative result, (ii) the ability of RT-PCR to detect the antibody neutralized NDV that cannot be detected through VI. In the present study the findings of RT-PCR from pooled tissue samples agree with Ghom *et al*. (2010), Haque *et al*. (2010) and Singh *et al*. (2005), while contradicts with Creelan *et al*. (2002) which might be due to a change in nature and quality of samples tested. Moreover, two-step RT-PCR provides another advantage of rapidity as the viral RNA was extracted directly from tissue homogenate and tracheal swab suspension without propagation in embryonated eggs and detected within 24 hrs as compared to VI (3-4 days). Another disadvantage of VI was the requirement of a large number of specific pathogen free (SPF) eggs while dealing with a large number of samples during the outbreak.

As the clinical signs and gross lesions are considered as the first indicator of a disease, so in the present study the efficacy of clinical diagnosis was compared with RT-PCR results and analyzed statistically. The nervous signs alone or in combination with respiratory and enteric signs were significantly related ($P<1.00$) with RT-PCR while a non-significant relationship ($P<0.08$- 0.48) of enteric and respiratory signs was found with molecular diagnosis. From these results it can be concluded that the nervous signs were the true indicator of ND during this outbreak and respiratory and enteric signs observed might be due to the other closely related viral and bacterial infections like chronic respiratory disease, avian influenza, mycotoxicosis, fowl cholera and necrotic enteritis.

From the above study it can be concluded that despite the difficult collection and handling, the pooled tissue samples are the best specimen for the detection of NDV than the tracheal swabs. Furthermore, RT-PCR can be used as a method of choice for the rapid and accurate diagnosis of ND during emergency conditions. As being accurate, less laborious and less time consuming, the RT-PCR should be standardized and used in the local laboratories in the context of Pakistan.

There is no conflict of interest among the authors and all authors agree to the above mentioned data.

REFERENCES

Aldous, E.W. and D.J. Alexander. 2001. Detection and differentiation of Newcastle disease virus (Avian paramyxovirus type I) Avian Pathol. 30:117-128.

Alexander, D.J. 1997. Newcastle disease in other avian paramyxoviridae infections. In: Diseases of poultry, 10th ed. Iowa State Uni. Press, Ames, Iowa, USA. pp. 541-570.

Alexander, D.J. 1998. Newcastle disease and other avian paramyxoviruses. A laboratory manual for the Isolation and Identification of Avian Pathogens. 4th ed, American Association of Avian Pathologists, University of Pennsylvania, Kennett Square, PA, USA. pp:156-163.

Alexander, D.J. 2003. Newcastle disease, other avian paramyxoviruses, and pneumovirus infection. In: Y.M. Shaif, H.J. Barnes, J.R. Glisson (Eds.), Disease of Poultry,12th ed. Blackwell, Oxford, UK. pp.75–100.

Alexander, D.J., J.G. Bell, R.G. Alders. 2004. A Technology review: Newcastle disease. Food and Agriculture Organization (FAO) of United Nations, Rome.

Bancroft, J. D. and M. Gamble, 2007. Theory and Practice of Histopathological Techniques. 5th ed. Churchill Livingstone, London. pp.85-100.

Anonymous, 2012. Pakistan Poultry Association Press release: 25 April 2012. Lahore, Pakistan.

Brown, C., D.J. King and B.S. Seal. 1999. Pathogenesis of Newcastle disease in chicken experimentally infected with viruses of different virulence. Vet Pathol. 36:125-132.

Creelan, J.L., D.A. Graham, S.T. McCullough, 2002. Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction. Avi Pathol. 31:493-499.

De Leeuw, O. and B. Peeters. 1999. Complete nucleotide sequence of Newcastle disease virus evidence for existence of a new genus within the subfamily *Paramyxovirinae*. J. Gen. Virol. 80:131-136.

Doyle, T.M. 1927. A hitherto unrecorded disease of fowls due to a filter-passing virus. J. Comparat. Pathol. Therapeutics 40:144-169.

Gohm, D.S., B. Thur. and M.A. Hofmann. 2000. Detection of Newcastle disease virus in organs and feaces of experimentally infected chickens using RT-PCR. Avi Pathol. 29:143-152.

Haque, M. H., M.T. Hossain, M.T. Islam, M.A. Zinnah, M.S.R. Khan and M.A. Islam. 2010. Isolation and detection of Newcastle disease virus from field outbreaks in broiler and layer chickens by Reverse transcriptase polymerase chain reaction. Bangl. J. Vet. Med. 8:87-92.

Iqbal, M., K .Mahboob, M. Zulifiqar, A. Haq, G. Nabi and R. Tubssum. 2003. Production of hyperimmune serum against Newcastle disease virus (NDV) in rabbits. Pak. J. Vet. Res. 1:22-25.

Jestin, V. and A. Jestin, 1991. Detection of Newcastle disease virus RNA in infected allantoic fluid by *in vitro* enzymatic amplification (PCR). Arch. Virol. 118:151-161.

Kaleta, E.F., C. Baldauf, 1988. Newcastle disease in free living and pet birds. In: D.J. Alexander (ed.). Newcastle Disease, Kluwer Academic Publisher, Boston, MA. USA. pp.197-246.

Kirkland, P.D. 2000. Virulent Newcastle disease virus in Australia: in through the 'back door'. Austral. Vet. J. 78:331-333.

Kommers, G.D., D.J. King, B.S. Seal, C.C. Brown. 2003. Pathogenesis of chicken-passage Newcastle disease viruses isolated from chickens, wild and exotic birds. Avian Dis 47:319-329.

Kraneveld, F.C. 1926. A poultry disease in the Dutch East Indies. Nederlands-Indische Bladen voor Diergeneeskunde 38:448-450.

Lamb, R.A. and D. Kolakofsky. 1996. Paramyxoviridae: The viruses and their replication. Virology, 3rd ed. Lippincott, Philadelphia. pp.1177-1204

MAFF/ADAS. 1984. Manual of Veterinary Investigation. 3rd ed. Reference Book Her Majesty's Stationary, Office London.

Mayo, M.A., 2002. A summary of taxonomic changes recently approved by ICTV. Arch Virol. 147:1655-1663.

Nguyen, T.T., H.J. Know, I.H. Kim, S.M. Hong, W.J. Seong, J.W. Jang and J.H. Kim. 2012. Multiplex nested RTPCR for detecting avian influenza virus, Infectious bronchitis virus, and Newcastle disease virus. J. Virol. Methods 188:41-4.

OIE, (2012). Newcastle disease. Manual of diagnostic tests and vaccines for terrestrial animals. 7th ed, Office international des Epizooties, Paris. pp.555-573.

Oldonia, I., C.C. Brown, D.J. King, S. Samald, and B.S. Seal, 2005. The use of in situ hybridization and immunohistochemistry to study the pathogenesis of various Newcastle disease virus strains and recombinants in embryonated chicken eggs. Mic. Pathol. 39:69-75.

Roy, P. and A.T. Venugopalan. 1999. Dot-enzyme linked immunosorbent assay for demonstration of Newcastle disease virus infection. Comp. Immu. Micro Infec. Dis. 22:27-31.

Singh, K., N. Jindal, S.L. Gupta, A.K. Gupta, and D. Mital. 2005. Detection of Newcastle disease virus genome from the field outbreaks in poultry by Reverse Transcriptse Polymerase Chain Reaction. Int. J. Poult. Sci. 4:472-475.

Smietanka, K., Z. Minta and K. Domanska-Blicharz. 2006. Detection of Newcastle disease virus in infected chicken embryos and chicken tissues by RT-PCR. Bull. Vet Inst. Pulawy. 50:3-7.

Stevens, J.G., R.M. Nakamura, M.L. Cook and S.P. Wilczynski. 1976. Newcastle-disease as a model for

paramyxovirus-induced neurological syndromes—pathogenesis of respiratory-disease and preliminary characterization of ensuing encephalitis. Infect. Immun. 13:590-599.

Susta, L., P. Miller, C. Afonso, C. Brown. 2010. Clinico-pathological characterization in poultry of three strains of Newcastle disease virus isolated from recent outbreaks. Vet Pathol. 48(2):349-360.

Wakamatsu, N., D.J. King, D.R. Kapczynski. 2006. Experimental pathogenesis for chickens turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002-2003. Vet. Pathol. 43:925-933.

Westbury, H. 2001. Commentary, Newcastle disease virus: an evolving pathogen. Avi. Pathol. 30:5-11.

Wise, M.G., D.L. Suarez, B.S. Seal, J.C. Pedersen, D.A. Senne, D.J. King, D.R. Kapczynski and E. Spackman. 2004. Development of a Real-Time Reverse-Transcription PCR for detection of Newcastle Disease Virus RNA in clinical samples. J. Cli. Micro. 42:329-338.

Wu, H.Y., S.H. Chiou, J.H. Shien, P.C. Chang and H.K. Shieh. 1999. Detection of protein and nucleic acids of Newcastle disease virus in *Eimeria acervulina*. Avi. Pathol. 28:441-445.