Pak. j. life soc. Sci. (2013), 11(3): 225-232 E-ISSN: 2221-7630;P-ISSN: 1727-4915

Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Pathotyping of Newcastle Disease Virus using Multiplex Reverse Transcription Polymerase Chain Reaction and Pathological Studies in Naturally Infected Broiler Chicks

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ARTICLE INFO

Received: Aug 27, 2013
Accepted: Nov 25, 2013
Online: Nov 26, 2013

Keywords
Broiler
Field survey
Multiplex RT-PCR
Natural infection
Newcastle disease virus
Pathotyping

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ABSTRACT

Multiplex RT-PCR was standardized to diagnose Newcastle disease (ND) in field survey of 50 poultry (Broiler) farms suspected for ND, under local laboratory conditions. Clinical signs and gross lesions of infected broilers were recorded. Respiratory signs were observed at 50 (100%) farms visited. Enteric signs were seen at 45 (90%) farms. Nervous signs including torticollis, paralysis of wings and legs, blindness and depression were found at 15 (30%) farms. Among these 15 farms, 10 (20%) farms were found to be infected with velogenic NDV through multiplex RT-PCR.. Congestion and mucosal haemorrhages were seen throughout the mucosa of trachea at 35 (70%) farms visited along with mucoid and catarrhal secretions trapped inside the tracheal tract. Congested lungs were found at 30 (60%) farms. Marked spleenomegaly was observed at 15 (30%) farms. Lymphoid hyperplasia with haemorrhages and necrosis was seen in spleen. Visceral organs including trachea, lungs and spleen were collected for histopathological studies and virus isolation. Virus isolation was carried out into 9-day- old chicken embryonated eggs. Multiplex RT-PCR was conducted for the identification of different pathotypes of ND virus, using a set of three primers (P1, P2 and P3). A total of 10 (20%) isolates having HA titre of 1:1024 (3 isolates), 1:512 (5 isolates), 1:256 (2 isolates) and showing amplicons of 204 bp were pathotyped as velogenic NDV and other 20 (40%) samples which resulted the amplification of 204bp and 364bp amplicons simultaneously, were categorized as mesogenic NDV. The conditions of multiplex RT-PCR optimized in this study can be used for rapid identification and differentiation of NDV pathotypes from field outbreaks or experimental pathogenesis of ND.

INTRODUCTION

Newcastle disease (ND) is an extremely infectious malady of poultry having considerable fiscal shock on world poultry production (Alexander and senne, 2008). It occurs due to ND virus, which belongs to *Paramyxoviridae*, subfamily Paramyxovirinae and genus Avulavirus (Wakamatsu et al., 2006). Serious outbreaks of ND are reported even in vaccinated flocks (Nakamura et al., 2008). ND is considered enzootic in Pakistan and poses great risk to the economy of the country which is mainly dependent on agriculture, livestock and poultry. Poultry industry is flourishing day by day in Pakistan and has contributed directly or indirectly in the employment of about 1.5 million people. Poultry meat shares about 19% of the total meat production in the country (Ghafoor et al., 2010).

ND has destroyed poultry industry several times in Pakistan by its different pathotypes which are commonly known as lentogenic, mesogenic and velogenic depending upon their virulence. These pathotypes produce mild, moderate and highly acute types of infection in birds. There are further two pathotypes of velogenic NDV which are known as

viscerotropic velogenic (VVNDV) and neurotropic velogenic (NVNDV) on the basis of their symptoms (Alexander, 2003).

In outbreaks in chickens due to the velogenic pathotypes, different symptoms include increased respiration, torticollis, weakness, paralysis of legs and wings, greenish diarrhoea, muscle tremors, ending with prostration and death. Neurotropic velogenic form of NDV in chickens is obvious by rapid onset of severe respiratory problem followed by neurologic signs but diarrhoea is usually absent (Ojok & Brown, 1996).

Mesogenic pathotype of NDV generally results respiratory distress in field infections. Generally, clinical signs do not develop in adult birds due to lentogenic viruses but may result severe respiratory problems in susceptible birds (Alexander & Senne, 2008).

Mean death time (MDT), Intravenous pathogenesity index (IVPI) and Intracerebral pathogenesity index (ICPI) have been used for pathotyping of NDV (Roy & Venugopalan, 1999). All these techniques are time consuming, labor demanding and sometimes not decisive (Seal et al., 1995).

Other techniques like PCR enables rapid identification of pathotypes of the virus and is the sensitive and the specific method (Awan et al., 2012; Ayaz et al., 2013). Multiplex RT-PCR using oligonucleotide primers having NDV genomic RNA, is one of the fast, reliable and reproducible molecular techniques that can be used for the identification of pathotypes of NDV (Ali & Reynolds, 2000). Multiplex RT-PCR is one of the latest molecular techniques which can be used as an alternative of *in vivo* methods of virulence assessment (MDT, ICPI, IVPI). The advantages of this technique over the *in vivo* methods for pathotyping of NDV are that this technique is rapid, accurate and the results are reproducible. Keeping in view the advantages of this technique, the present project was designed to study clinico-pathological changes produced in broilers naturally infected with NDV and to standardize the multiplex RT-PCR technique for the rapid and confirmatory diagnosis and pathotyping of NDV under local laboratory conditions.

MATERIALS AND METHODS

Field survey

A field survey of 50 broiler farms suspected for ND was conducted in 2009-2010 in Faisalabad, Pakistan. The number of broilers at each farm varied from 1000-3000 and the age of the broilers varied from 25-35 days. All the farms were vaccinated against ND by using vaccinal strain (La Sota). Three diseased birds showing typical symptoms of ND from each farm were selected randomly. Clinical signs of infected broilers were recorded. Post mortem of birds was conducted to study gross lesions. Trachea, lungs and spleen were used for histopathological studies. Histopathology was done following the procedure, described by Bancroft and Gamble (2002).

Virus Isolation

Virus isolation was conducted in embryonated chicken eggs as done earlier by Smietanka et al. (2006). Tissues of trachea, lungs and spleen of the sampled birds from each farm were pooled together to represent as one field isolate and triturated in tissue homogenizer after diluting in normal saline @ 10% w/v followed by centrifugation at 1000 g at 4°C for twenty minutes. After centrifugation, supernatant was collected and antibiotics were added to stop any bacterial and fungal contamination. Each field isolate was inoculated @ 0.2 ml into five, 9-day-old embryonated chicken eggs via allantoic cavity route and incubated at 37°C. The eggs having dead embryos within first 24 hrs were discarded and remaining embryos which died between 24-72 hrs were harvested and kept overnight at 4°C. Allantoic fluid of each field isolate was collected under aseptic conditions and preserved at -20°C till next use.

Serological identification of the virus

Haemagglutination activity of allantoic fluid was tested by spot agglutination and haemagglutination test. Confirmation of NDV was done by haemagglutination inhibition test as described by MAFF (1984) by using specific hyperimmune sera which was raised by inoculating La Sota strain into five adult chicks. Blood samples from the inoculated birds were collected at different time intervals to check their antibody titre against NDV by HI test and after having highest antibody titer, blood was collected aseptically without anticoagulant in sterilized centrifuge tubes. Serum was isolated from the clotted blood.

Multiplex RT-PCR

After serological identification of NDV, positive samples were pathotyped using multiplex RT-PCR technique. Viral RNA was extracted from allantoic fluid. Isolation of RNA was carried out using GF-1 viral nucleic acid extraction kit (Vivantis, GF-RD-100) as described by the manufacturer. RNA extracted by this method was stored at -20 ºC. Following the extraction of RNA, reverse transcription of viral RNA was carried out using RevertAid^{TM} first strand cDNA Synthesis kit (Fermentas) to synthesize complementary DNA (cDNA). Two mixtures, mixture-I and mixture-II were prepared to synthesize cDNA. Mixture-I was prepared by mixing 5µl of RNA per sample, random hexamer primer 2µl per sample and DEPC treated water 3µl per sample and incubated at 70ºC for 5 min and cooled at room temperature. Mixture-II was prepared upto 10µl, consisting of 5x RT buffer 5ul per sample, bovine serum albumin 1µl per sample, 10mM dNTPs Mix 1µl per sample, RevertAid^{TM} M- MuLV Reverse transcriptase 1µl per sample and 200 U/µl

DDT 2µl per sample. Mixture-I and II were mixed and incubated at 37ºC for 1 hour. cDNA synthesized was stored at -70ºC till further use.

Primers

Three primers, primer 1 (P1), primer 2 (P2) and primer 3 (P3) specific to the F-gene sequence of NDV were used. Keeping P1 common for the three pathotypes, P2 was used for mesogenic and velogenic pathotypes and P3 for lentogenic and mesogenic pathotypes. The base pairs of these primers were as follows;

P1: 5' GCA GTC AAC ATA TAC ACC TCA TC 3' (Forward, 191-213).

P2: 5'ATA AAG CGT CTC TGC CTC CT 3' (Reverse , 380-399).

P3: 5' TCA GAG ACC TCI TGC ACA GCC 3' (Reverse, 535-555).

PCR Procedure

After the reverse transcription reaction was completed and cDNA was synthesized, 30 µl PCR mixture was added into 20 µl of cDNA solution. PCR mixture of 30 µl was prepared by mixing 1 µl of P1 (10 pmol /µl), 1 µl of of P2 (20 pmol/µl), 1 µl of P3 (20 pmol/µl), 4 µl of 2.5 mM of dNTPs, Taq polymerase 0.3 µl, 10x PCR buffer 5 µl and 17.7 µl of DEPC- dd H_2O. A total of 50 µl solution (30 µl PCR mixture and 20 µl cDNA) was run in thermocycler (Peq-lab primus 25 USA) for denaturing, annealing and extension. The following conditions were optimized for denaturing, annealing and extension of DNA using the live vaccinal (La Sota) strain of NDV and then samples were subjected to multiplex RT-PCR using these optimized conditions. For the denaturation of DNA, temperature was set at 94°C for 4 min following 10 cycles of denaturation at 94°C for 30 sec. For annealing, temperature was set at 50°C for 30 sec following 72°C for 45 sec for extension. The next 20 cycles of denaturation were conducted at 94°C for 30 sec, annealing and extension at 55°C for 30 sec and 72°C for 45 sec, respectively. Last cycle was executed at 72°C for 10 min. Results were examined by electrophoresis of 2% agarose gel and visualized in Gel documentation system (Wealtec Dolphin Doc, USA) taking 50bp DNA ladder as reference value.

RESULTS

Clinical Signs

Clinical signs observed during this field survey were categorized into three categories; respiratory, nervous and enteric signs. Respiratory signs were observed at 50 (100%) farms visited. Coughing was observed in birds at 20 (40%) farms. Enteric signs were seen at 45 (90%) farms visited. Nervous signs including torticollis, paralysis of wings and legs, blindness and depression were found at 15 (30%) farms (table 1).

Table 1: Clinical Signs found in naturally infected broilers at 50 ND suspected Farms and their Percentage

Clinical Signs	No. of Farms (Out of 50)	Percentage (%)
Off Feed	45	90
Diarrhoea	45	90
Nasal discharge	50	100
Gasping	50	100
Dyspnoea	50	100
Coughing	20	40
Depression	15	30
Torticollis	15	30
Wing and leg paralysis	15	30
Blindness	15	30
Cyanosis of comb & wattles	35	70
Oedema of head	25	50

Gross Lesions

Marked congestion and mucosal haemorrhages were seen throughout the mucosa of trachea of naturally infected broilers at 35 (70%) farms visited. Mucoid and catarrhal secretions were also found trapped inside the tracheal tract (Fig. 1). Congestion was found in lungs at 30 (60%) farms (Fig. 2). Membranes of air sacs of these birds were thick and opaque containing some exudates. Marked spleenomegaly was observed at 15 (30%) farms (Fig. 3).

Histopathological Studies

The histopathological changes seen in trachea included necrosis and congestion around tracheal rings. Congestion and necrosis were also observed in the skeletal muscles (Fig. 4). Lungs showed proteinaceous material in alveolar spaces (Fig. 5). There were also found inflammatory zones in lungs (Fig. 6). Lymphoid hyperplasia with haemorrhages and necrosis was seen in spleen (Fig. 7).

Virus Isolation

Allantoic fluid was collected from embyonated eggs and tested for agglutination activity by spot agglutination test, haemagglutination test (HA) and confirmed for NDV by haemagglutination inhibition test (HAI). Spot agglutination test showed 37 (74%) isolates positive while 13 (26%) isolates were found negative. Out of 37 positive isolates, 30 (60%) isolates were confirmed for NDV in HI test.

Multiplex RT-PCR

After confirmation of NDV isolates by haemagglutination inhibition test, positive samples were pathotyped by multiplex RT-PCR. RNA isolated from 10 (33%) out of 30 positive NDV isolates showed amplicons of 204bp. While the simultaneous amplification of 204bp and 364bp amplicons was resulted with RNA isolated from other 20 (66%) positive NDV isolates as shown in Fig 8 & 9.

In the figure 8, "M" is the molecular weight marker which is 50bp DNA ladder. Lane 1 is the –ve control. Lane 2 shows a RT-PCR amplicon of 204bp which

Table 2: Haemagglutination Titre of Allantoic fluid of Virus Isolates

No. of Isolates	Haemagglutination Titre									
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
03	+	+	+	+	+	+	+	+	+	+
05	+	+	+	+	+	+	+	+	+	-
02	+	+	+	+	+	+	+	+	-	-
11	+	+	+	+	+	+	-	-	-	-
09	+	+	+	+	+	-	-	-	-	-
07	+	+	+	+	-	-	-	-	-	-

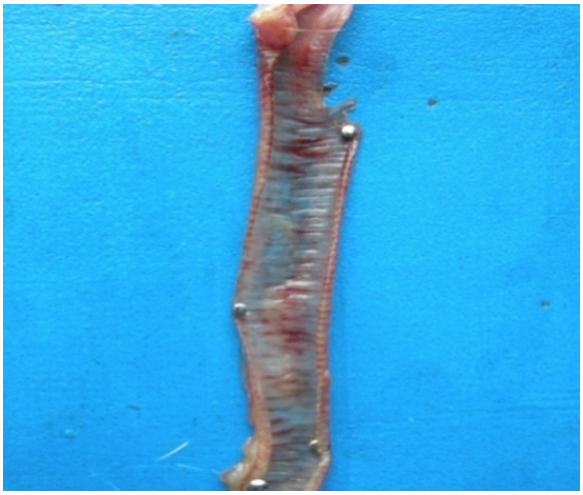

Fig. 1: Haemorrhages in Trachea in ND suspected broilers.

Fig. 3: Spleenomegaly in ND suspected broilers

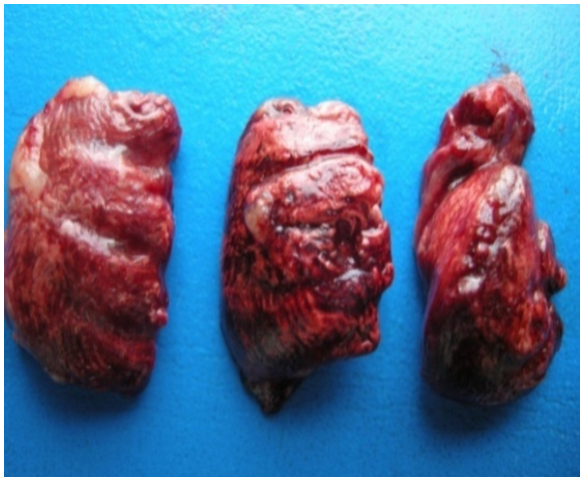

Fig. 2: Congestion of Lungs in ND suspected broilers

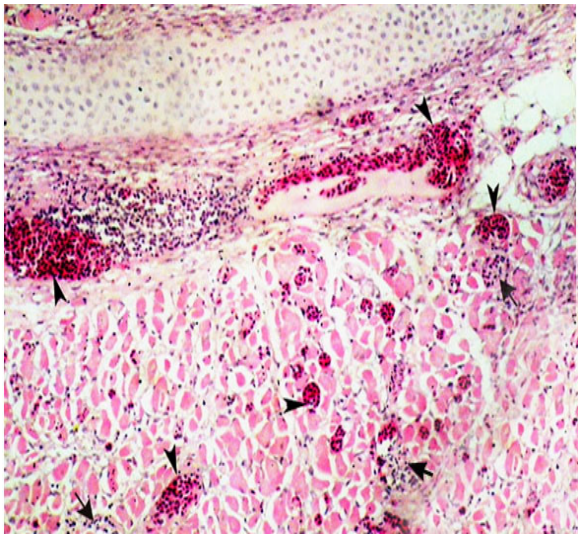

Fig. 4: Photomicrograph of Trachea showing congestion (►) and necrosis of skeletal muscles (→)

results only with RNA isolated from velogenic NDV. Lane 3 & 4 shows RT-PCR amplicons at 204bp and 364bp which results with RNA isolated from mesogenic NDV. Lane 5, 6 & 7 shows no RT-PCR amplicons as some negative samples in HI test were also run along with positive samples to confirm the results.

In the figure 9, L1 is the -ve control. L2 and L7 show a RT-PCR product of 204bp. While L3, 4, 5 & L6 show RT-PCR product of 204bp and 364bp.

DISCUSSION

Newcastle disease is a highly dangerous disease of poultry worldwide. It has devastating effects on poultry industry in Pakistan. In Pakistan, poultry farmers suffer from heavy losses due to ND every year, inspite of vaccination against ND. Different pathotypes of ND

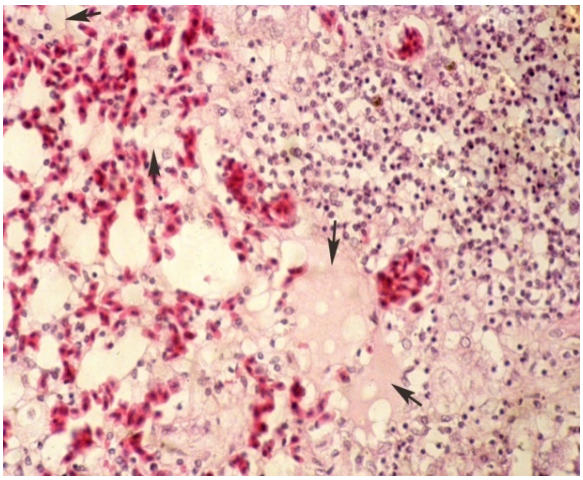

Fig. 5: Photomicrograph of Lung showing exudates (proteinacious material) in alveolar spaces.

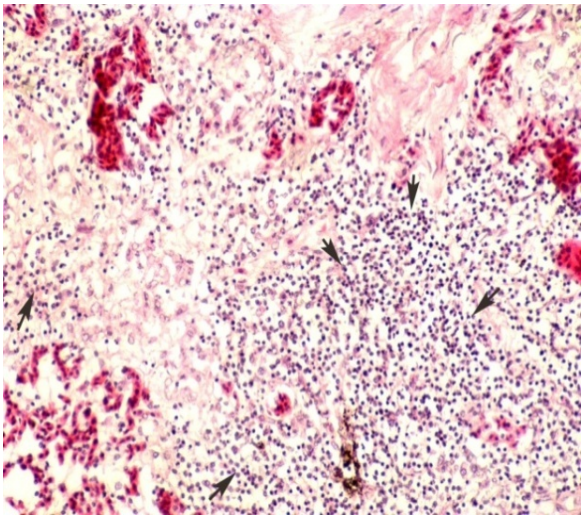

Fig. 6: Photomicrograph of Lung showing Inflammatory zones.

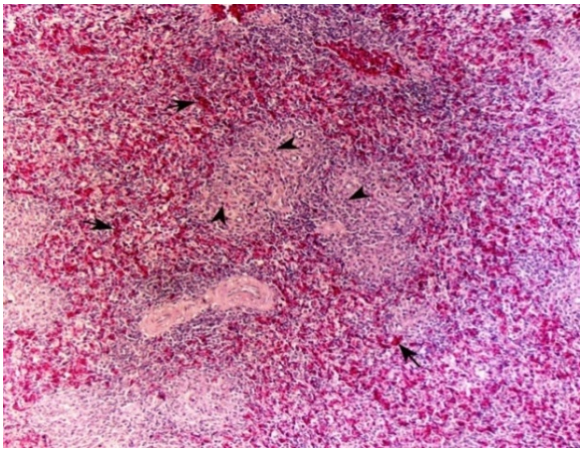

Fig. 7: Photomicrograph of Spleen showing haemorrhages (→) and necrosis (►).

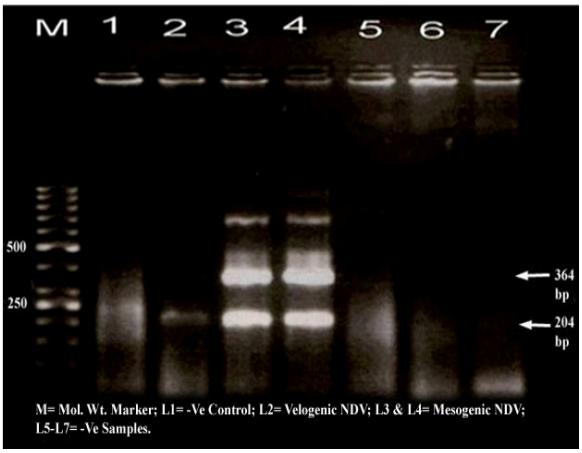

Fig. 8: Agarose gel showing the RT-PCR results of NDV field isolates.

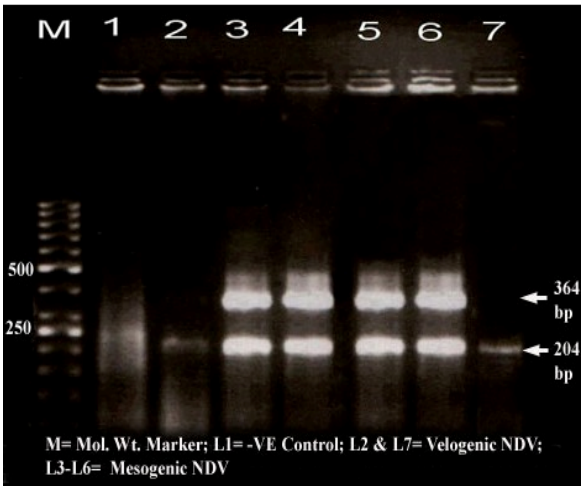

Fig. 9: Agarose gel showing the RT-PCR results of NDV field isolates.

are prevailing in Pakistan, causing heavy damage to the flourishing poultry industry as observed by Ghafoor et al. (2010). Timely diagnosis of the disease is of utmost importance for effective control measures. Mostly conventional methods including MDT, IVPI and ICPI are used in Pakistan to differentiate the different pathotypes of ND. These methods take much time and sometimes the results are not accurate as reported earlier by Nanthakumar et al. (2000). for timely diagnosis of the disease and to know which pathotype has invaded the farm, a method is required which could help in such identifications more precisely, accurately and within short period of time. Molecular methods including RT-PCR are being used for accurate, precise, quick diagnosis and pathotyping of different infectious diseases including ND as reported by Farkas et al. (2007). Keeping in view the above said facts, the present study was planned to differentiate different pathotypes of ND using multiplex RT-PCR from field outbreaks in broilers.

A field survey was conducted and fifty farms showing clinical symptoms more or less similar to ND were selected. Three samples from each farm were selected randomly among the severe diseased birds. Clinical signs were recorded and post mortem was conducted to observe gross lesions. Trachea, lungs and spleen were used for histopathological studies. Among the 50 farms visited, respiratory signs were seen at all the farms. Nervous signs including torticollis, paralysis of legs and wings, blindness and depression were found at 15 (30%) farms. Out of these 15 farms showing nervous signs, 10 (20%) farms were found to be infected with velogenic NDV through multiplex RT-PCR. Brown et al. (1999) observed depression, paralysis of legs and conjunctivitis in experimental inoculation of viscerotropic and neurotropic velogeniec NDV in chickens. Similar symptoms were observed by Nakamura et al. (2004) and Wakamatsu et al. (2006) in experimentally infected chickens by velogenic NDV.

Membranes of air sacs of the birds were thick and opaque containing some exudates. Edematous fluid was seen in alveoli of lungs by Shahzad et al. (2011) in broilers, experimentally infected with velogenic strain of NDV. Wakamatsu et al. (2006) observed air sacculitis, lymphoid depletion and encephalitis in chicken. Congestion was found in lungs at 30 (60%) farms. Marked congestion and mucosal haemorrhages were seen throughout the mucosa of trachea of infected broilers at 35 (70%) farms visited. Mucoid and catarrhal secretions were also found trapped inside the tracheal tract. Piacenti et al. (2006) observed airsaculitis and tracheitis in turkeys experimentally infected with mesogenic and velogenic strains. Among the 15 (30%) farms showing spleenomegaly, 10 (20%) farms were found to be infected with velogenic NDV through multiplex RT-PCR. Susta et al. (2011) observed enlargement of spleen in chickens, experimentally infected with velogenic NDV. Lymphoid hyperplasia with haemorrhages and necrosis was seen in spleen. Spleenic lymphoid hyperplasia was observed in birds infected with mesogenic NDV isolates by 5 dpi by Brown et al. (1999). Allantoic fluid was used for virus isolation and its haemagglutination activity was tested by spot agglutination and haemagglutination test followed by confirmation of NDV by haemagglutination inhibition test. Spot agglutination test showed 37 (74%) isolates positive while 13 (26%) isolates were found negative. Out of 37 positive isolates, 30 (60%) isolates were confirmed for NDV in HI test. Siddique et al. (2005) and Mustafa et al. (2010) also used HA and HAI tests for the identification of ND.

Samples which were inhibited by known NDV antiserum in HI test were pathotyped by multiplex RT-PCR. Out of 30 field isolates, 10 (20%) isolates showing amplicons of 204 bp were pathotyped as velogenic NDV. The next 20 (40%) isolates which resulted the amplification of 204bp and 364bp amplicons simultaneously, were categorized as mesogenic NDV, in accordance with the previous reports on pathotyping of NDV through multiplex RT-PCR, where 204 bp amplicons are resulted only with the RNA isolated from velogenic NDV and a mixture of 204 bp and 364 bp amplicons results with the RNA isolated from mesogenic NDV, using the similar oligonucleotide primers as reported by Songhua et al. (2003).

The HA titters of the isolates which showed amplicons of 204bp and categorized as velogenic NDV, were recorded as1:1024 (3 isolates), 1:512 (5 isolates) and 1: 256 (2 isolates) while lower titres were observed for mesogenic isolates showing simultaneous amplicons of 204bp and 364bp. No lentogenic pathotype of NDV was recovered from the field isolates. Khan et al. (2005) observed that the haemagglutination titers of NDV strains causing disease were ranged from 1:16 to 1:64 and haemagglutination inhibition titers was 1:256.

Clinical signs and gross lesions found at those farms which were found to be infected with velogenic NDV by multiplex RT-PCR, were similar to pathological changes produced by velogenic NDV in experimental pathogenesis as reported by different workers. The other 20 farms which were found to be infected with mesogenic NDV by multiplex RT-PCR showed more or less similar pathological changes as seen in farms infected with velogenic NDV, except for necrotic ulceration of intestine which was seen in velogenic infected farms.

Out of 50 broiler farms visited during the study, 30 farms were found infected with NDV pathotypes. The remaining 20 (40%) farms showing pathological changes in respiratory, enteric and nervous systems may be infected with other infectious diseases like Avian Influenza, Fowlpox, Infectios Laryngotracheitis, Fowl cholera, Mycoplasmosis or Infectious bronchitis.

The conditions of multiplex RT-PCR optimized in this study can be used for rapid identification and differentiation of NDV pathotypes from field outbreaks or experimental pathogenesis of ND under local laboratory conditions.

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