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RESEARCH ARTICLE

DETECTION OF NEWCASTLE DISEASE VIRUS (NDV) ISOLATES FROM POULTRY BY USING REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

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ABSTRACT

Newcastle disease (ND) an infectious highly contagious avian disease causing severe economic losses to poultry worldwide and controlled by vaccination. In spite of regular vaccination a number of sporadic outbreaks are observed in vaccinated as well as non vaccinated birds and constant threat to poultry since last few years. Isolation and identification of circulating Newcastle disease virus isolates are crucial for the accurate diagnosis of the virus strains, which could be valuable for developing better prophylactic measures. This study was under taken to assess the prevalence of NDV in poultry farms. Among all the total of 39 collected field samples from various poultry farms the NDV was detected in seven field samples by RT-PCR. All the seven field samples yielded a band of 1662 bp on amplification of complete coding region of fusion protein gene of NDV. The results of this simple assay clearly indicated that the NDV strains are circulated in a range of susceptible birds.

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INTRODUCTION

Poultry is one of the fastest growing segments of the agricultural sector and provide globally important sources of animal protein and are amongst the most intensively reared of all livestock species. The causative agent of the disease is Newcastle disease virus (NDV) belongs to the genus *Avulavirus* within the family *Paramyxoviridae* (Mayo, 2002). Poultry farmers across the globe face devastating effects that poultry and the economic decline that is consequent to the loss of poultry and poultry products is also brought on by viral diseases. Newcastle Disease (ND) is fatal and still top ranked economically important poultry disease and also a major threat to poultry industry facing millions of dollars losses every year worldwide (Khan *et al*., 2011, Haquee *et al*., 2010, Waheed *et al*., 2013, Susta *et al*., 2010, Narayanan *et al*., 2010). ND infection has been reported from a wide variety of birds with varying degree of susceptibility (Kaleta and Baldauf, 1988).This disease can have devastating effects on the poultry industry due to the high morbidity and mortality (up to 100%) associated with virulent strains of the virus**.** The RT-PCR has established its position top over the other confirmatory tests in the field of diagnosis of Newcastle disease (Singh *et al*., 2005, Krzysztof *et al*., 2006). The speed of the diagnosis can be considerably increased by using molecular biology methods like PCR (Aldous and Alexendar, 2001). This emphasizes the importance of research concerning poultry viruses. Diseases of poultry are the major concern, both locally and on an international scale. Therefore, investigation for the early detection and identification of the virus is imperative and may lead to the eventual eradication of the poultry viruses.

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MATERIALS AND METHODS

Sample collection

A total of 39 clinically suspicious sick and dead birds believed to be infected with Newcastle disease were collected from various locations in Chittoor district during 2012 to 2014 (Table 1). The birds were transported to department of pathology, Sri Venkateswara Veterinary University, Tirupati and subjected to post-mortem examination and the representative tissue samples from the respiratory system (trachea), gizzard, liver, and proventriculus and heart tissues were collected. The tissue samples were placed in phosphate buffer saline (PBS) containing antibiotics were transported to the department of virology, Sri Venkateswara University, Tirupati and stored at -80^{0}C for further use.

Table 1. Collection of NDV suspected samples from different poultry farms

S.No	Place of collection	Sample Name	Layer/ Broiler	Age (days)	No. of samples collected
		ND-1			
1	Padipeta	ND-2	layers	50	4
		ND-3			
		ND-4			
		ND-5	layers	45	3
2	Mallamgunta	ND-6			
		ND-7			
		ND-8			
3	Karakambadi	ND-9	layers	41	4
		ND-10			
		ND-11			
		ND-12			
4	Cherlopalli	ND-13	layers	60	4
		ND-14			
		ND-15			
5	Rangampeta	ND-16	broilers	39	2
		ND-17			
		ND-18			
6	Battamdoddi	ND-19	broilers	40	3
		ND-20			
		ND-21			
7	Panjani	ND-22	broilers	35	3
		ND-23			
8	Lingapuram	ND-24	broilers	28	2
		ND-25			
		ND-26			
9	Kondasamudram	ND-27	broilers	32	4
		ND-28			
		ND-29			
		ND-30			
10	Penchupalli	ND-31			
		ND-32	broilers	31	4
		ND-33			
		ND-34			
11	Agraharam	ND-35	broilers	35	2
12	Ramapllli	ND-36	broilers	30	2
		ND-37			
13	Chowdepalli	ND-38	broilers	28	2
		ND-39			
		Total no of samples			39

Isolation of Newcastle disease virus from embryonated chicken eggs (ECE)

The tissue samples (trachea, gizzard, liver, proventriculus, and heart) were pooled and homogenized by using sterile mortar and pestle with liquid nitrogen. The homogenized samples were centrifuged at 8000 rpm for 10min was done in order to eliminate debris and bacteria to the possible extent. The supernatant fluid was mixed with antibiotics penicillin (2000 IU/ml), streptomycin (2 mg/ml), gentamycin (50 ug/ml) and mycostatin (1000 units/ml) for one hour before and the tissue homogenate was passed through 0.22µ syringe filter (Sartorius) and 0.2 ml of filtrate was inoculated into the allontoic cavity of 10 days old embryonated chicken eggs for isolation.

Source of embryonated chicken eggs

Fertile eggs were obtained from Sri Balaji hatcheries, Chittoor and the department of poultry science, college of veterinary science, Tirupati. These eggs were cleaned and swabbed with 70 % alcohol and kept an egg incubator at 85 % humidity for 10 days. Tenth day old chicken embryos were used for cultivation of virus.

Cultivation of virus

The Newcastle disease virus was cultivated in ten day old chicken embryos inoculated by the allontoic sac route OIE (2012) standard procedure.

Molecular identification of NDV

Viral RNA isolation

Viral genomic RNA was isolated from haemagglutination test positive allontoic fluid samples using Trizol reagent (Fermentas, Germany) according to the manufactures protocol. 250 µl of allontoic fluid were taken in a sterile eppendorf tube and added 250 ul Trizol reagents was added and then incubated at room temperature for 5 min for dissociation of nucleoproteins. 0.2 ml of chloroform was added and shaken vigorously for 15 seconds and allowed to stand for 2-5 minutes at room temperature. The upper separated aqueous phase was transferred to a fresh tube and 0.5 ml of iso-propanol was added per 1 ml of tri reagent used. The mixture was shaken vigorously and incubated at room temperature for 10 min and centrifuged at 12000X g for 10 minutes to pellet the RNA. The RNA pellet was washed with 1 ml of 75% ethanol by

centrifuged at 7500X g for 5 min. The RNA pellet was briefly dried and resuspend in 20-30 ul of RNase-free DEPC treated water and stored at -80 ◦C until further use.

Reverse transcription (RT)

The reverse transcription was carried by Sambrook and Russell (2001**)** method. Brifly, 4 µl of viral RNA, 2.0 µl of random hexamer primer and 6.5 µl of Double distelled water were taken into an RNase free 0.2 ml eppendorf tubes and incubated the mix at 70^0C for 5 minutes and immediately chilled on ice. Mix the following reagents such as 4.0 µl of 5X reaction buffer, 2.0 µl of 10mM dNTPs mix and 0.5 µl of ribonuclease inhibitor was added, mixed well with pipette and incubated for 5 min at 37^0 C. Finally, 200 U of M-MuLv reverse transcriptase (Fermentas, USA) was added to the reaction mixture and mix gently and centrifuge briefly and kept into a thermo cycler for cDNA synthesis and incubate 10 minutes at 25^0C followed by 60 minutes at 42^0C. The reaction was stopped by heating the mixture for 10 minutes at 70^0C.

Primer design and synthesis

Primers to amplify the fusion protein gene of NDV were designed using the softwares, NCBI blast, Bio-edit and Primer 3. Initially the conserved region among the isolates of NDV were identified by NCBI and Bio-edit and later the primer design was made on where the sequence does not show any intragenic loops or secondary structures and dimmers. Care was taken to keep G, C residues at 3^1 ends of the primer, required G and C concentration and Tm value. The designed primers were synthesized commercially from MWG, Bangalore, India. A 100 picomoles primer stock was prepared with sterile DEPC treated water and the working concentration (10 pm) was made from stock aliquoted and stored at -20^0C.

Reverse transcription polymerase chain reaction (RT-PCR)

The reverse transcribed cDNA was then subjected to RT-PCR. The PCR was performed using a total volume of 50 µl reaction mixture containing 5.0 µl 10X buffer, 4.0 µl 25mM $MgCL_2$, 2.0 µl 10 mM dNTP, 3.0 µl NDV Forward primer (NDV KK F), 3.0 µl NDV Reverse primer (NDV KK R), 0.5 µl Taq DNA polymerase (Fermentas), cDNA 1.5 µl and 31µl DEPC water was added to the PCR tube and mixed with micropipette. The tubes were immediately transferred to the thermo cycler (Corbett, UK) and cycling conditions were programmed as follows 94^0 C for 5 minutes followed by 30 cycles of 94^0 C for 40 seconds, 55^0 C for 1 minute, 72^0 C for 1.00 minute and final elongation of 72^0C for 7 minutes.

Analysis of PCR products by Agarose gel electrophoresis

The amplified PCR products were subjected to agarose gel electrophoresis and the band pattern was recorded using UV gel documentation system (UVP, USA).

RESULTS AND DISCUSSION

The representative samples collected from field among all the forty two samples only seven samples (ND-5, ND-11, ND-14, ND-19, ND-20, ND-28, ND-32) showed congested embryos and hemorrhages on the body surface (Figure 1) of all the inoculated embryos were observed after 4 days of post inoculation and gave positive results had a single band of 1662 bp in the RT-PCR and the remaining samples were not showed any band pattern on 1% agarose gel (Figure 2) and to evaluate the specificity of PCR products by size of the amplicons has been verified by NDV Lasota (Vaccine strain) used as positive control and Infectious Bursal disease virus Georgia strain (vaccine strain) used as negative control.

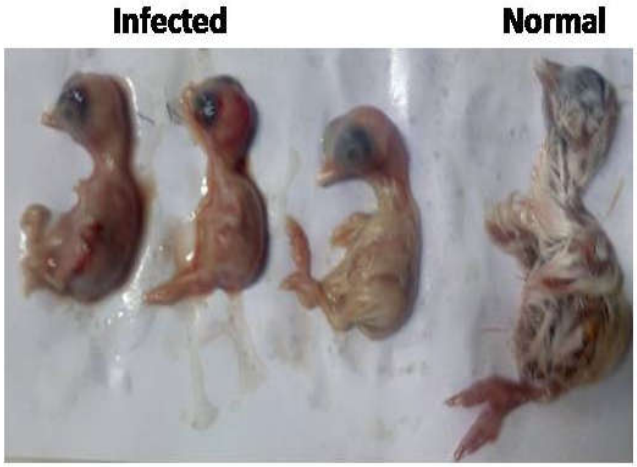

Figure 1: NDV infected chicken embryos

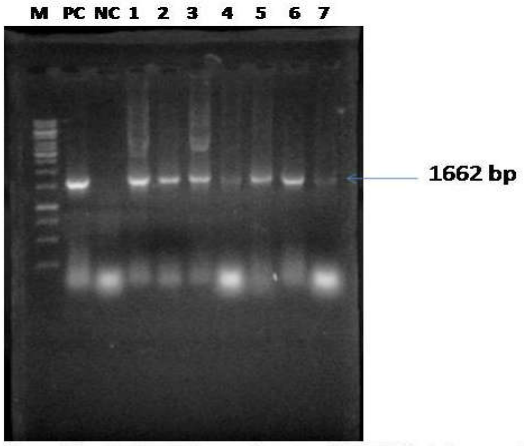

Figure 2 : Amplified RT-PCR products of NDV field samples
M : 1kb DNA marker (fermanatas)
PC : Positive control
NC: Negative control
1-7: Field samples

In conclusion, seven isolates of NDV were successfully isolated and identified from vaccinated chickens by using RT-PCR. This preliminary study it will fast, sensitive and diagnostic tool enable the veterinary personnel to implement any emergent actions and also help ful for generate epidemiological data as well as in formulation of vaccination strategy for effective control of the disease under field conditions.

Further pathotypic and genotypic characterization these NDV field isolates are now in progress.

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