SHORT COMMUNICATION

A sensitive haemadsorption technique based RT-PCR for concentration and detection of Newcastle disease virus from clinical samples and allantoic fluid

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Abstract The present study describes the exploitation of haemadsorption (HAD) property of the Newcastle disease virus (NDV) for the development of a novel sensitive HAD technique based RT-PCR for detection of NDV from clinical samples of virus infected experimental birds. The NDV propagated allantoic fluid from the infected embryonated chicken eggs or supernatant of the processed clinical samples (tissue triturate, cloaca and tracheal swabs) from the experimentally infected birds were added with chicken red blood cells (RBC) to adsorb the virus on RBC's surface. The virus adsorbed RBCs were subjected to trizol method of RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) for detection of NDV. The HAD based RNA extraction showed better yield of 700–900 ng RNA and when subjected to RT-PCR detection revealed a 100 times higher sensitivity than the conventional RNA extraction and RT-PCR detection system. This could be an alternate technique which can be exploited in low NDV load situations in clinical samples.

Keywords Haemadsorption · RNA extraction · Newcastle disease · RT-PCR

Newcastle disease virus (NDV), an avian paramyxovirus -1 (APMV-1) of the family *Paramyxoviridae* and Genus *Avulavirus*, causes a highly contagious and fatal disease known as Newcastle disease (ND) which affects most species of birds worldwide, and is one of the most economically important diseases of poultry [3]. Because of the severe nature of the disease and having transboundary implications as well as associated socio-economical consequences, ND is considered as a notifiable avian disease which must be reported to World Organization for Animal Health (Office Internationale des Epizootics) [10, 12, 15]. The OIE has adopted an expanded definition of ND with the inclusion of alternative criteria of virulence based on the demonstration of a characteristic pattern of amino acid residues (directly or by deduction) in the region of the fusion protein cleavage site of NDV fusion (F) gene [1, 2], thereby enabling molecular-based techniques to become integrated into the diagnosis of NDV. At present, RT-PCR-based techniques for the detection and typing (pathotyping and genotyping) of APMV-1 is becoming common in diagnostic laboratories [6, 9, 10]. The direct tissue RT-PCR has been reported to be less sensitive as compared to embryonated chicken egg inoculated allantoic fluid based RT-PCR, since the viral load in the affected organs/tissues may vary widely [5]. Recently, the techniques of haemadsorption (HAD) of ND viruses on chicken RBC surface followed by de-adsorption and subsequent RNA extraction has been reported to increase the sensitivity of RT-PCR detection system in clinical samples [14]. In this procedure, the de-adsorption of different strains or isolates of NDV from chicken RBC required different conditions and chemical concentration. Hence, a highly variable de-adsorption protocol is needed for detection of each NDV strain or isolates which is a limiting factor for its use in routine diagnostic purposes of NDV infection and disease

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outbreak investigations. In this context, we exploited the HAd property of NDV for developing a novel diagnostic protocol without the use of de-adsorption step to increase the sensitivity of RT-PCR detection system for NDV.

The three archived NDV isolates (NDV/Peafowl/Haryana/IVRI-037/12, NDV/Peafowl/UP/IVRI-024/12 and NDV/Peafowl/Delhi/IVRI-0022/12) maintained in Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India were used for the study purpose. The chicken NDV positive clinical samples (cloacal and tracheal swab) and tissues (brain, spleen, trachea and lung) available in the Avian Diseases Section were also used.

The freeze dried NDV isolates were reconstituted in one ml of phosphate buffered saline (PBS, pH 7.2). The inoculums were prepared for virus propagation as per standard protocol [10]. The reconstituted 0.2 ml of inoculums were inoculated into 9–11 day-old specific pathogen free (SPF) embryonated chicken eggs (Venkateshwara Hatcheries Private Limited, Pune, Maharashtra, India) through allantoic route, and the eggs were incubated at 37 °C till death or maximum period of 120 h, whichever was earlier. To check the viability of embryos, the eggs were candled every day and the dead embryos were chilled at 4 °C for overnight and the allantoic fluids were tested for hemagglutination (HA) activity [10]. After 120 h, all the remaining live embryos were collected and chilled at 4 °C for overnight and the allantoic fluids were tested for HA activity. The harvested allantoic fluids were tested for confirming the presence of ND virus by hemagglutination inhibition (HI) test with LaSota specific serum [10] and also by RT-PCR.

The NDV positive experimental samples viz., individual tracheal swabs, cloacal swabs and infected tissues (brain, spleen, trachea and lungs) were processed separately, mixed in PBS (pH 7.2) and triturated thoroughly to make homogenate suspensions (20 % w/v). The suspensions were then centrifuged at 1200 rpm for 15 min and the clear supernatants were harvested for use in haemadsorption and RT-PCR studies.

The chicken blood was collected from five birds aseptically in a disposable syringe and transferred to vacutainer containing EDTA (BD, India) as anticoagulant. The blood was centrifuged at 1500 rpm for 10 min and the plasma and buffy coat were removed with a pipette. After washing with equal volume of sterile PBS for three times, the chicken RBC suspension was collected.

In order to get good quantity of NDV, 3 ml of allantoic fluid obtained during NDV propagation in embryonated chicken eggs at first passage or 3 ml supernatant obtained from the processed clinical tissues and swabs of NDV infected birds was added with 50 µl of pelleted chicken RBCs (washed three times with equal volume of sterile

PBS, pH 7.2). This mixer was incubated in a shaker incubator (C24KC, New Brunswick Scientific, USA) at 37 °C for 20 min at 145 rpm and then centrifuged at 1500 rpm for 5 min to harvest the RBC pellet. After decanting most of the supernatant, approximately 300 µl of the left over supernatant with RBC pellet was mixed thoroughly and used for extraction of RNA by TRIzol method.

For RNA extraction, 300 µl of RBC pellet obtained after haemadsorption was mixed with TRIzol[®] reagent (Invitrogen, USA) and RNA was extracted as per the manufacturer's instructions. The extracted RNA was used to synthesize cDNA by using random hexamer (MBI Fermentas, USA). The reverse transcription (RT) for first strand synthesis was carried out by using RevertAid H minus (MMuLV-RT) (MBI Fermentas, USA) in a standard 20 µl reaction mixture. The RT mixture was made with 5 µl of total RNA, 1 µl of random hexamer primer, 4.0 µl of 5X RT buffer, 2.0 µl of dNTP mix (10 mM), 0.5 µl of RNase inhibitor and 1.0 µl MMuLV-RT enzyme (200 U/µl). The reverse transcription reaction was carried out with incubation of RT mixture for cDNA synthesis at 25 °C for 10 min, 42 °C for 60 min and 70 °C for 10 min. The RT-PCR was performed using NDV fusion gene published primers (F₁- GCAGCTGCAGGGATTGTGGT, F₂- TCTTTGAGCAGGAGGATGTTG, amplifying a 356 bp size) [13] to amplify F₀ cleavage site. The RT-PCR reaction was optimized in a standard 25 µl reaction mixture containing 3.0 µl of cDNA, 12.5 µl of DreamTaq PCR Master mix 2X (Thermo scientific, India), 1.0 µl each of Forward and Reverse primer (10 pmol/µl), and 7.5 µl of nuclease free water. The RT-PCR cyclic conditions were initial denaturation at 95 °C for 4 min followed by 35 cycles of 94 °C for 40 s, 57 °C for 50 s, 72 °C for 50 s and final extension at 72 °C for 5 min. The RT-PCR products were analyzed by electrophoresis in 1.5 % agarose gel stained with ethidium bromide (0.5 µg/ml) and amplification bands were observed and recorded in a Gel Doc System (Alphainnotech co., San Leandro, CA).

The diagnostic sensitivity of HAd based RT-PCR was compared with direct tissue sample, swabs, and allantoic fluid based conventional RT-PCR for detection of NDV, by serial dilution of each virus containing fluids. For this purpose, 4 HA allantoic fluids, supernatants of processed tracheal and cloacal swabs, and supernatants of processed pooled tissues were serially diluted tenfold. The direct conventional RT-PCR was performed with the RNA extracted from 300 µl of each of these tenfold dilutions for detection of NDV. For HAd based RT-PCR, 2 ml of each of these tenfold dilutions was used to concentrate the virus separately as described in the HAd method for virus concentration, RNA was extracted and RT-PCR was performed for detection of NDV. The diagnostic sensitivity

limit of both the HAd based RT-PCR and conventional RT-PCR was compared. Apart from the diagnostic sensitivity testing of the tenfold dilution of the fluids, direct tissue sample sensitivity testing of the HAd based RT-PCR and direct conventional RT-PCR was also evaluated for detection of NDV in experimentally infected clinical tissues of birds. For this purpose, 22 NDV positive clinical tissue samples [brain ($n = 8$ samples), trachea and lungs ($n = 8$ samples), and spleen ($n = 6$ samples)] that were collected from the earlier experimental studies, presented with characteristic histopathological lesions for NDV, were subjected to HAd based RT-PCR and conventional RT-PCR assays for detection of NDV.

All three Indian—peafowl origin NDV isolates, propagated in embryonated chicken eggs and the harvested allantoic fluids, were found positive for HA and HI. The HAd based RNA extraction from the allantoic fluids showed visible RNA pellets and better RNA yield of 700–900 ng (260/280 and 260/230 values were between 1.8 and 2.0). The conventional RNA extraction procedure of allantoic fluids showed invisible or feebly visible RNA pellets and RNA yield of 50–100 ng. Similarly, the haemadsorption based RNA extraction from the processed tracheal and cloacal swabs and the homogenated tissues yielded good quantity of RNA as comparable with to conventional RNA extraction procedure.

The diagnostic sensitivity of the HAd based RT-PCR for NDV detection was between 10^{-3} and 10^{-5} dilutions (Fig. 1), for conventional RT-PCR it was only between 10^0 and 10^{-2} dilution (Fig. 1). The diagnostic sensitivity of the

HAd based RT-PCR with the 4 HA allantoic fluids was observed highest for up to 10^{-5} dilutions, while for the cloacal swabs, tracheal swabs and the infected tissues it was up to 10^{-4} , 10^{-3} and 10^{-3} , respectively. Compared to HAd based RT-PCR, the conventional RT-PCR detected NDV in 4HA allantoic fluids up to 10^{-2} dilution, while the cloacal swabs, tracheal swabs and the infected tissues detected the virus up to 10^{-2} , 10^{-1} and 10^0 dilutions, respectively (Table 1).

The HAd based RT-PCR detected 20 samples positive for NDV out of 22 NDV clinical tissue samples, whereas in conventional RT-PCR only 14 samples were found positive for the virus. The diagnostic viral positivity of the brain, trachea and lungs, and the spleen in HAd based RT-PCR procedures were 7/8, 8/8, and 5/6, respectively, while in conventional RT-PCR procedure it was 4/8, 7/8, and 3/6, respectively (Table 2). The diagnostic specificity of the HAd based RT-PCR was 90.9 percent, whereas with conventional RT-PCR it was only 63.6 percent in NDV positive clinical samples.

NDV, a notifiable transboundary avian disease remains a constant threat to the poultry industry. The early and accurate detection of NDV infection is of prime importance for effective control of the disease. The usual problem encountered in NDV diagnosis are less number of virus excretion in samples, uneven distribution of NDV in tissues and use of labor consuming in-humane diagnostic procedures (emroyonated chicken egg inoculation etc.). The protocol used for RNA extraction may also affect the outcome of RT-PCR on clinical specimens. In commercial

Fig. 1 Sensitivity of haemadsorption based RT-PCR for detection of diluted tenfolds, **a** Allantoic fluid, **c** Cloacal swabs **e** Tracheal swabs; **g** Infected tissues. Sensitivity of conventional RT-PCR is to detect the

NDV, diluted tenfolds; **b** Allantoic fluid, **d** Cloacal swabs; **f** Tracheal swabs; **h** Infected tissues

Table 1 Comparative sensitivity of haemadsorption based RT-PCR and conventional RT-PCR for detection of NDV in clinical samples of virus infected birds and infected allantoic fluid, diluted tenfolds

Clinical samples	Sensitivity by haemadsorption based RT-PCR	Sensitivity by conventional RT-PCR
Allantoic fluid (4HA)	10^{-5}	10^{-2}
Cloacal swabs	10^{-4}	10^{-2}
Tracheal swab	10^{-3}	10^{-1}
Infected tissues	10^{-3}	10^0

Table 2 Comparative sensitivity of haemadsorption based RT-PCR and conventional RT-PCR for direct detection of NDV in clinical tissues of virus infected birds

Samples	Sensitivity by haemadsorption based RT-PCR	Sensitivity by conventional RT-PCR
Brain	7/8	4/8
Lungs and trachea	8/8	7/8
Spleen	5/6	3/6

RNA extraction kits care should be taken in selecting the most appropriate or validated samples for analysis [10]. The developed HAd based RNA extraction protocol has several advantages over other conventional RNA extraction procedures. One of the advantages of this procedure is, the allantoic fluid has more fluid part and less cellular, viruses, and harvesting good quantity of RNA in conventional Trizol method is difficult. In this procedure, one could concentrate 2 ml of allantoic fluid containing virus into 300 μ l of RBC pellet adsorbed NDV and at the same time RNA from RBC [4] also increases RNA concentration and it is easy to make RNA pellet. The RNA extraction from human RBC has also been demonstrated by few workers [4, 8]. RNA extraction by Trizol method was done in human RBC for detection of West Nile Virus [11]. Formalin treated chicken RBC was used to adsorb the avian influenza viruses from environmental samples and the adsorbed RBC are processed for RNA extraction [7]. Yi and Liu [14] used the chicken RBC adsorption and de-adsorption technique of RNA extraction for NDV to prevent contamination in faecal and tissue samples which will hinder in RT-PCR. The problem in adsorption and de-adsorption technique is that de-adsorption of different NDV strains or isolates are difficult since variable de-adsorption conditions and chemical concentration for different NDV strains or isolates; there is a need for de-adsorption protocol standardisation for each strain or isolates. The de-adsorption based RT-PCR is of limited use in routine diagnosis of NDV infection, outbreak investigation.

The other advantage is, in tracheal, cloacal swabs and faecal samples the NDV load is highly variable and

conventional RNA extraction has less sensitivity. The hemadsorption based RNA extraction protocol increase the sensitivity of the RT-PCR test. The RNA extraction and RT-PCR from direct clinical samples showed less sensitivity compared to embryonated chicken egg grown allantoic fluid. [5]. In majority of the outbreaks there will be less chance for availability of suitable clinical samples; hence there is a need for concentrating the fewer viruses in tissue. The conventional RNA extraction protocol uses less quantity of tissue trituate supernatant and chance for less viral load in tissue trituate. The newly developed haemadsorption based RT-PCR was sensitive, no need to de-adsorb NDV and the direct RBC adsorbed NDV can be used in Trizol method of RNA extraction. The developed new method of RNA extraction alleviates the problem of fewer viruses in samples, and there is no need for concentrating the virus by ultracentrifuge or other procedures. This developed technique could be a better alternate procedure for outbreak investigation and surveillance programmes. Further studies are needed in large scale, with different pathotype of NDV and in different species of birds to validate this technique for routine diagnosis of NDV.

The newly developed hemadsorption based RNA extraction procedure improved the diagnostic sensitivity of RT-PCR over conventional RNA extraction based RT-PCR in detecting NDV in clinical samples, thus the developed technique of RNA extraction alleviates the problem of fewer viruses in samples, and there is no need for concentrating the virus by ultracentrifuge or other procedures.

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