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Rafia Maqbool
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Shakil A Wani
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Asifa Wali
Faculty of Fisheries, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Zahid Kashoo
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Pervaiz Dar
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology Of Kashmir (SKUAST-K) India

Sabia Qureshi
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Isfaqul Hussain
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Aazima Shah
Division of Veterinary Pathology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology Of Kashmir (SKUAST-K) India

Parvaiz Sikander
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology Of Kashmir (SKUAST-K) India

MY Ganaie
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Saba Bukhari
Division of Animal Genetics and Breeding, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

Correspondence
Rafia Maqbool
Division of Veterinary Microbiology & Immunology, Faculty of Veterinary Science & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (SKUAST-K) India

RT-PCR based assay for detection of Newcastle disease virus isolated from poultry in Kashmir

Rafia Maqbool, Shakil A Wani, Asifa Wali, Zahid Kashoo, Pervaiz Dar, Sabia Qureshi, Isfaqul Hussain, Aazima Shah, Parvaiz Sikander, MY Ganaie and Saba Bukhari

Abstract
Newcastle disease (ND) caused by a virulent strain of Avian paramyxovirus-1(vAPMV-1), is included in List A of the Office International des Epizooties and is a highly contagious disease of poultry. ND remains a major problem in existing or developing poultry industries. Confirmatory diagnosis requires isolation and detection of the virus. The present study was carried out to detect the Newcastle disease virus by RT PCR based assay isolated from poultry in Kashmir. 9 out of 10 disease outbreaks suspected for ND were positive. The clinical and postmortem lesions were characteristic of ND. The virus was isolated on the chicken embryo fibroblast (CEF) cell line. The virus was able to replicate easily in the cell line without supplementing with any trypsin-like protease. The detection was carried out by amplification of 356 bp hypervariable region of Fusion protein gene with F_0 cleavage site by RT-PCR which is regarded as a major determinant of pathogenicity.

Keywords: Fusion protein gene, Reverse transcriptase PCR

Introduction
Newcastle disease (ND), a poultry disease that causes severe outbreaks resulting in huge economic losses, is caused by *Newcastle disease virus* (NDV) [1]. NDV is a single stranded negative sense, non-segmented RNA virus, which is a virulent strain of avian paramyxovirus type 1 (APMV-1) serotype of the genus *Avulavirus*, belongs to subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales* [2, 3]. The disease has a worldwide prevalence including in India and is seen affecting many species of birds causing huge economic loses to poultry industry due to high morbidity and mortality associated with virulent strains of the virus [4, 5, 6]. There are 9 serotypes of APMV, but all isolates of Newcastle Disease Virus (NDV) belong to serotype 1 (APMV-1) [3], therefore NDV is synonymous with APMV-1. The APMV-1 viral genome, approximately of 15Kb, is composed of 6 genes encoding 6 structural proteins- fusion (F), nucleoprotein (NP), matrix (M), phosphoprotien (P), RNA polymerase (L), and hemaglutinin-neuraminidase (HN) [7, 8, 9]. Two additional proteins are encoded by RNA editing of the P protein, namely V and W. The V protein is known to have an inhibitory effect on the alpha/beta interferon response in an avian host [10].
There are different pathotypes of NDV in chickens: velogenic (high virulence), mesogenic (moderate virulence), and lentogenic (low virulence) [11]. The pathogenicity of NDV is determined primarily by cleavage of F protein by host cellular proteases [12, 13].
Historically, diagnosis of APMV-1 infection relies on the detection of the infectious agent by virus isolation in embryonated eggs or cell cultures [9]. At present, virus isolation is the prescribed test for international trade [14] and remains the method of choice for confirmatory diagnosis or as the gold standard method for the validation of other techniques [1, 15, 16]. The first documented attempt to detect APMV-1 by reverse transcription polymerase chain reaction (RT-PCR) in allantoic fluids of embryonated fowl eggs dates back to 1991 [17]. Since then a variety of laboratory protocols ranging from gel-based RT-PCR to real-time RT-PCR, restriction enzymes –based procedures, and rapid sequencing has been developed and published [18].

Materials and methods
A total of 10 suspected outbreaks of Newcastle Disease virus infection in Kashmir valley were attended. The samples collected from the dead birds included tracheal swabs, cloacal swabs, caecal tonsils, spleen, brain, proventriculus and Payer's patches.

The samples were preserved in 50% glycerol saline at -20 ºC or directly in TRIzol reagent (Sigma, USA) at – 80 ºC. The filtered homogenate of suspected tissue in PBS was inoculated in CEF cell line for replication. RNA was extracted from the infected cell culture suspension using TRIzol Reagent. Complementary DNA (cDNA) synthesis was carried out using random hexamer primers and Revert-Aid First-Strand cDNA Synthesis kit. A primer set targeting the hypervariable region of Fusion protein gene [19] was used to detect NDV by RT-PCR. PCR assay was performed in a 25 µl total reaction volume in a 0.2 ml PCR tube on Master cycler gradient PCR machine (Eppendorf, USA) with Nuclease Free Water 13.5µl, 10x PCR Buffer 2.5µl, 25mM $MgCl_2$ 2.5 µl, 25mM dNTP mix 0.2 µl, Forward primers (5pM) 0.5 µl, Reverse primers (5 pM) 0.5 µl, Taq DNA Polymerase 0.3 µl, cDNA (2 µg/20 µl) 5 µl. At the end of the run, the amplification was checked by Agar gel electrophoresis.

Results and discussion

Clinically, live birds showed nervous symptoms, diarrhoea, respiratory rales and ruffled feathers, whereas dead birds on postmortem revealed respiratory and gastrointestinal involvement. Dead birds showed petechial hemorrhages on proventriculus, catarrhal exudates in the intestines, hemorrhagic caecal tonsils, hemorrhagic intestinal epithelium with ulcer formation, hemorrhagic Payer'spatches and swollen spleen [Fig1-6]. The infectivity of the suspected samples in CEF was seen in 9 out of the 10 outbreaks. The infectivity in cell line was sluggish in the first and second passage but from the third passage, the cytopathic effects (CPE) could be visualized and during the fourth and fifth passage, the effect was rapid and the monolayer started detaching within two days post inoculation. The cytopathic effects included characteristic rounding of the cells, failure of cell adhesion, vacuolization in cells, cell fusion and clustering, syncytium formation and monolayer detachment. During the fifth passages the CPE was graded from 25% to 100%. At 72 h post inoculation the CPE was 75% indicated by rounding up of cells and monolayer detachment, whereas at 96 h post inoculation the CPE was 100%. [Fig7-10]. The virus was able to replicate easily in the culture which was not supplemented with any trypsin-like protease indicating the presence of virulent form of NDV. The need for trypsin in the cell culture media has been used as an indication of pathogenicity. In order for the F protein to become active, it must be cleaved by the secretory trypsin-like proteases. These types of proteases are limited to the mucosal membrane of respiratory and gastrointestinal tracts. The virulent strains of NDV are able to replicate systematically due to the presence of multiple basic amino acids at the cleavage site of the F protein which make it easier to be cleaved by non-trypsin-like proteases. This is also true for in *vitro* analysis where virulent NDV is able to replicate and cause plaques in cell culture system lacking trypsin like proteases. Low virulent strains are not able to replicate systematically due to the limited availability of trypsin-like proteases [20, 21, 16].

In 9 out of 10 suspected outbreaks, RNA extracted from infected CEF cell suspension on RT- PCR amplified a fragment of the hypervariable region of F gene with the expected size (~356 bp) and confirmed the presence of NDV in the samples [Fig:11]. RT-PCR systems are usually being used to amplify a specific portion of the genome that will give added value; for example by amplifying part of the F gene that contains the F_0 cleavage site for detection of NDV and for assessing virulence by restriction digestion or amino acid sequencing of F_0 cleavage site [22].

Fig1: Haemorrhagic trachea

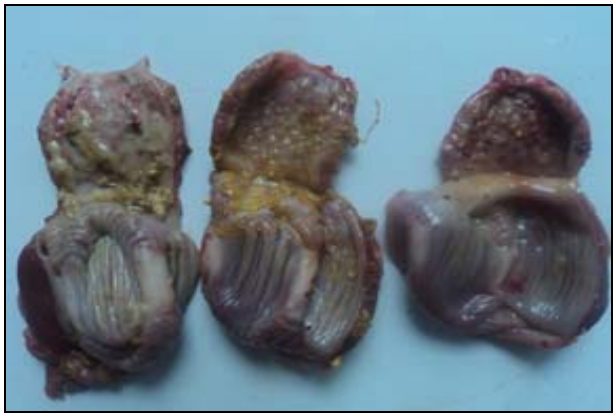

Fig 2: Petechial haemorrhages in proventriculus

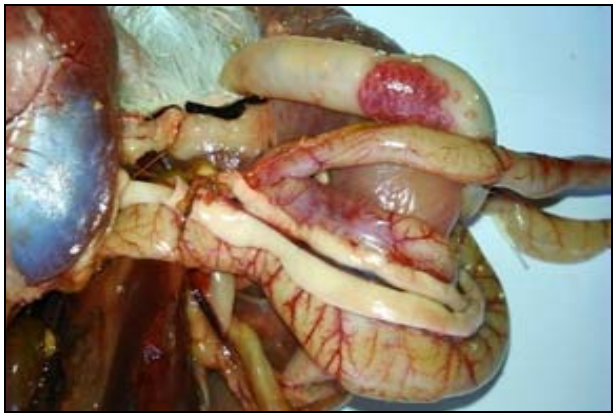

Fig 3: Ulcer intestine from serosal surface

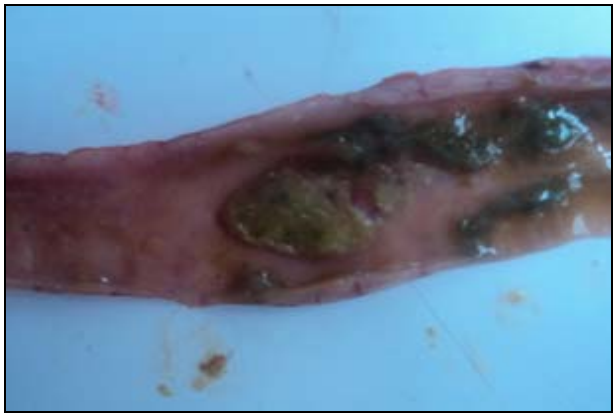

Fig 4: Ulcer intestine from mucosal surface

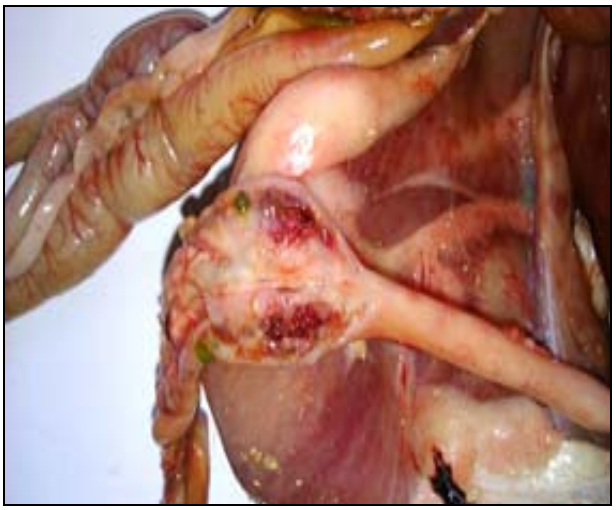

Fig 5: Haemorrhagic caecal tonsils

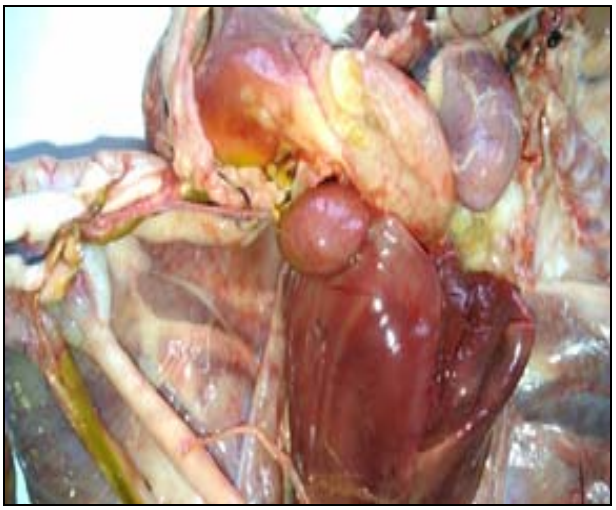

Fig 6: Swollen spleen

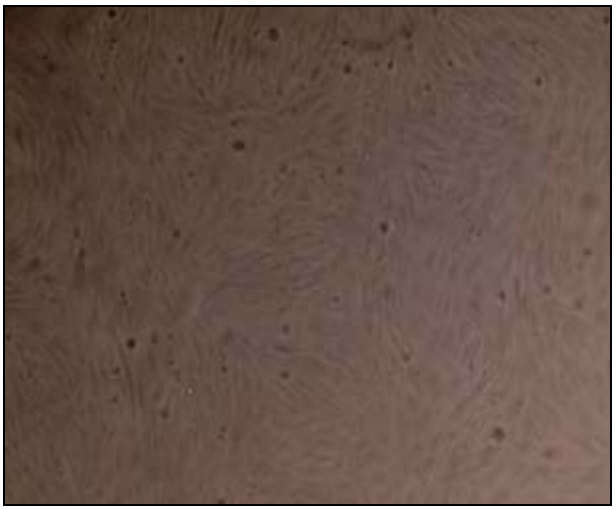

Fig 7: Chicken embryo fibroblast monolayer uninfected with spindle-shaped fibroblasts with characteristic cellular projections

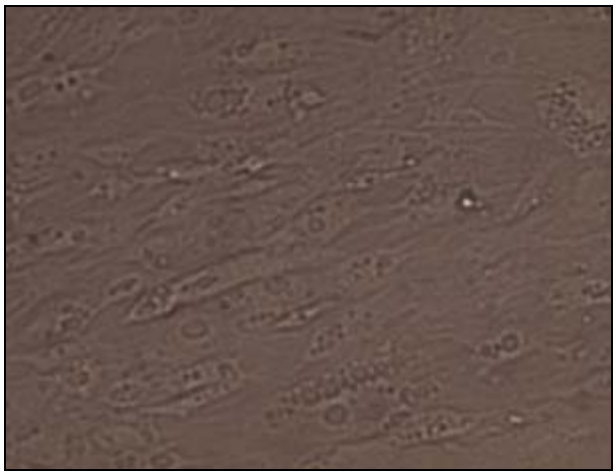

Fig 8: Chicken embryo fibroblast 48hrs Postinfection showing detaching of monolayer

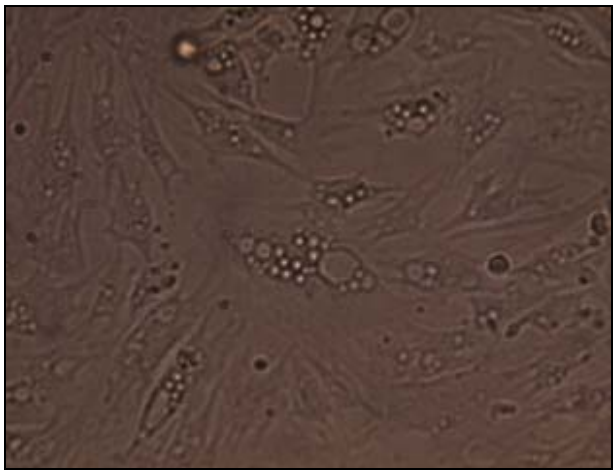

Fig 9: Chicken embryo fibroblast 72hrs Postinfection showing rounding, failure of cell adhesion, vacuolization, cell fusion and clustering and syncitium formation

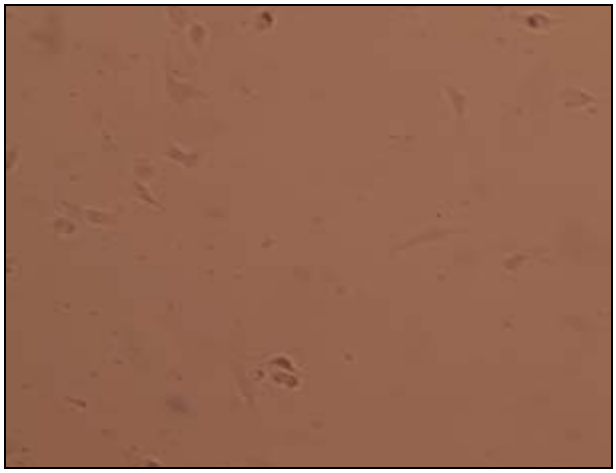

Fig 10: Chicken embryo fibroblast 96 hrs Postinfection showing complete disruption of monolayer

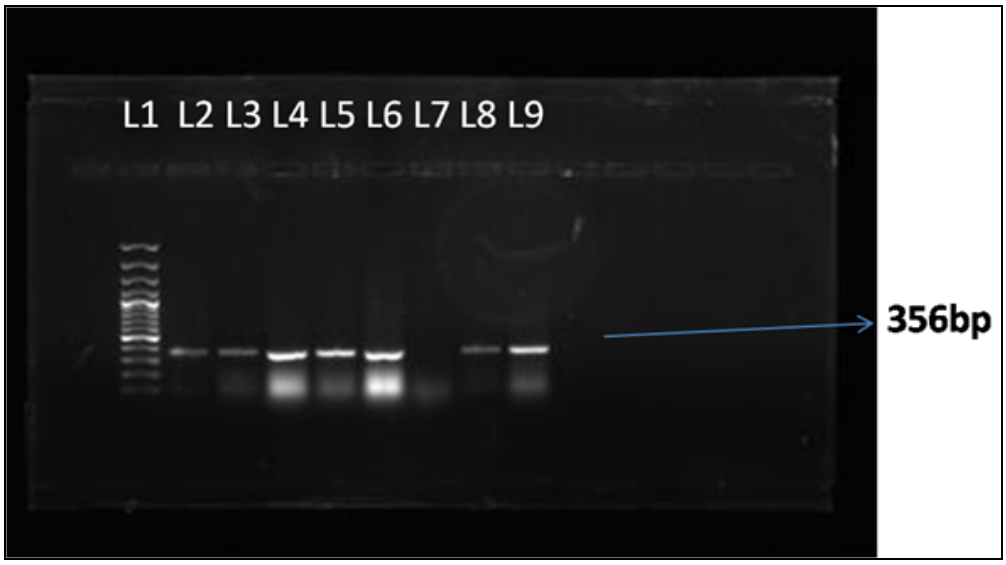

Fig 11: Fusion protein gene fragment (~356 bp) corresponding to the hypervariable region was amplified in the positive samples. L1= 100 bp DNA ladder, L2-L5, L8-L9= ND positive samples, L7= ND negative sample

Conclusion

Molecular detection of Newcastle disease followed by isolation is the gold standard for disease diagnosis. In nine out of ten outbreaks, virus was isolated from CEF cell line without supplementing trypsin indicating the presence of virulent strain circulating in Kashmir and then detection by RT-PCR**.**

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