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## Detection of Group D Avian Rotaviruses among Layer Poultry from Western India

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**Abstract:** Rotavirus is a major cause of gastroenteritis in infants and children worldwide and also in wide variety of mammalian and avian species. In the present study, 4 (7.84%) out of the 54 poultry faecal samples tested, were found positive by RNA-PAGE. The samples were of 60-65 weeks age layer poultry birds suffering from diarrhoea. All the PAGE positive samples resolved in to 11 segments with a migration pattern of 5:2:2:2 characteristic of group D avian rotavirus. All the positive samples were of short electropherotype in which segments 10<sup>th</sup> and 11<sup>th</sup> migrated very closely. Avian rotavirus samples subjected to VP4 and VP7 gene based RT-PCR did not amplified respective products. RNA-PAGE positive samples were further subjected to VP6 gene based RT-PCR amplification using specific primers. All the four positive samples yielded a specific product of 493 bp. The study reports first ever detection of Group D avian rotavirus in Western India.

**Key words:** Avian rotavirus, group D, RNA PAGE, RT-PCR, VP6

### INTRODUCTION

Rotavirus is a major cause of gastroenteritis in infants and children worldwide and also in wide variety of mammalian and avian species (Estes *et al.*, 1983). Avian rotaviruses were first detected in the United States and United Kingdom from diarrhoeic turkey poult (Mcnulty *et al.*, 1978; Bergeland *et al.*, 1997). Avian rotavirus causes enteritis of variable severity in poultry especially during the early stage of life (Tamechiro *et al.*, 2003). Huge economic losses are associated with diarrhoeal syndrome in birds, making this a major concern to poultry industry. Rotavirus belongs to family *Reoviridae*; possesses double stranded RNA genome consisting of eleven discrete segments. The inner core is composed of viral protein, VP6, encoded by gene segment 6. The outer capsid consists of two proteins, VP4, encoded by gene segment 4 and VP7, a glycoprotein encoded by gene segments 7, 8 and 9 depending upon the strain of rotavirus. Rotaviruses isolated from different species could be distinguished by the electropherotypes of dsRNA (Kalica *et al.*, 1978; Yashon and Schat, 1985). Avian rotaviruses known so far belong to group A, D, F and G, among which group A rotaviruses have been found to be the most common agents of diarrhoea in human, animal and avian species (Estes and Cohen, 1989; Saif, 1989). The genomic RNA segments cluster into four regions - I to IV. Mammalian group A rotaviruses have a migration pattern of 4:2:3:2, while avian group A rotaviruses have 5:1:3:2 and group

D have 5:2:2:2 electrophoretic migration pattern (Mcnulty *et al.*, 1981). Though some work has been done on study of prevalence of avian rotavirus in some parts of India (Wani *et al.*, 2003; Minakshi *et al.*, 2004). But such information of detection of avian rotavirus is lacking in Western India. The present investigation was aimed to find the prevalence of rotavirus in poultry in this part of the country and to standardize RT-PCR based detection of avian rotaviruses.

### MATERIALS AND METHODS

**Rotavirus reference strain:** Rotavirus reference strain RA1 belonging to group A Avian rotavirus provided by Dept. of Animal Biotechnology CCS HAU, Hisar was used as known positive control.

**Faecal samples:** Fifty four faecal samples were collected during October 2008 to March 2009 from 1-70 weeks age poultry birds from private and government layer farms. These birds were apparently healthy. However, some of them were passing loose faeces. All the faecal samples were collected in screw-capped plastic vials and stored at 4°C and immediately transferred to laboratory. The faecal samples were diluted in lysis buffer to make a 10% suspension, followed by centrifugation at 10000x g for 15 min to remove coarse particles and cellular debris. The clarified supernatants were stored at -20°C till further processing.

**Extraction of viral nucleic acid:** The extraction of dsRNA from 10% faecal suspension was done as described by Herring *et al.* (1982) with minor modifications. RNA pellet was air dried, dissolved in RNA PAGE sample buffer and stored at -20°C for further use.

**RNA-polyacrylamide gel electrophoresis (RNA-PAGE):** Discrete segmented RNA genome of rotavirus was analyzed by RNA-polyacrylamide gel electrophoresis (RNA-PAGE) using discontinuous buffer system without SDS as described by Laemmli (1970). RNA pellet was dissolved in RNA sample buffer and then samples were loaded in wells of 5% stacking and 7.5% resolving gels. The samples were resolved in to 11 segments by electrophoresis in Tris-glycine buffer. The gel was run at a constant voltage of 100 V till the dye just came out of the gel and later visualized after staining with silver nitrate method of Svensson *et al.* (1986) and then photographed. The gel was stored in 10% ethanol for further studies.

**Primers:** The consensus primers for VP4, VP6 and VP7 gene were synthesized as earlier described by Ito *et al.* (1995) were used in the present study for amplification of the respective segment.

**Reverse transcription polymerase chain reaction of VP4, VP6 and VP7 gene:** The RNA was extracted using the method of Chomoczynski and Sacchi (1987). Reverse transcription of viral RNA was performed using MMLV reverse transcriptase (Promega) in 25 µl reaction volume as per manufacturer's instructions. The amplification of VP4, VP6 and VP7 gene was carried out using respective forward and reverse primers (Table 1).

**cDNA synthesis:** The extracted RNA (2 µl) was taken in 0.2ml thin walled PCR tubes containing 1.5 µl of Dimethyl Sulfoxide (DMSO) and was denatured at 99°C for 5 min and immediately snap chilled on ice. To the PCR tube, 21.5 µl of reverse transcription reaction mix containing 100 ng of each forward and reverse primer (VP7 FP and VP7 RP, VP4 FP and VP4 RP and VP6 FP and VP6 RP) for respective gene, 100 mM dNTPs and 10x RT buffer and 0.5 µl of MMLV reverse transcriptase enzyme was added. After letting the primers to anneal at 25°C for 10 min, reverse transcription was carried out at 42°C for 60 min in thermal cycler. MMLV-RT was heat inactivated at 90°C for 5 min. The cDNA was stored at -20°C.

### Polymerase chain reaction

**VP4 gene:** PCR reaction was carried out in 25 µl volume containing 2 µl cDNA, 1.5 µl DMSO and 25 pmol of each primer. The mixture was heat-denatured at 99°C for 5 min, snap-chilled on ice and to this 5 µl of PCR mixture consisting of 2.5 µl 10 x PCRbuffer, 0.5 µl 10 mM dNTPs, 1.5 µl 25 mM MgCl<sub>2</sub> and 0.5 µl *Taq* polymerase (5 U/µl) (MBI Fermentas) was added. The PCR amplification was performed on thermal-cycler (Eppendorf Mastercycler) using one cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min and elongation at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were analyzed in 1% gel by agarose gel electrophoresis.

**VP6 gene:** Keeping reaction mixture same as mentioned for VP4 gene. The PCR amplification was performed on thermal-cycler using one cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 44°C for 1 min and elongation at 72°C for 2 min and final extension at 72°C for 10 min.

**VP7 gene:** Using reaction mixture same as described earlier. The PCR amplification was carried out with one cycle of initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 1 min, annealing at 44°C for 1 min and elongation at 72°C for 2 min and final extension at 72°C for 10 min.

### RESULTS AND DISCUSSION

Out of 54 poultry faecal samples tested, 4 (7.84%) were found positive by RNA-PAGE (Fig. 1). These samples belonged to 60-65 weeks age layer poultry birds which were apparently healthy. However, some of them were passing loose faeces. Earlier (Minakshi *et al.*, 2004) reported 23.19% positive samples from poultry farms in Haryana, India. Ahmed and Ahmed (2006) detected 2.43% prevalence of rotavirus infection in broiler chicken in Bangladesh. Higher rate of rotavirus infection have been documented by McNulty *et al.* (1984), where 70% of serum samples from broiler breeder were seropositive for rotavirus like virus. In this study, positive samples resolved in to 11 segments with a migration pattern of 5:2:2:2 characteristic of group D avian rotavirus. In RNA-

Table 1: List of oligonucleotide primers used for RT-PCR amplification of VP4, VP7 and VP6 genes of avian rotavirus

Gene	Primer	Sequence of primer	Expected product size
VP4	VP4 (1-20) (FP)*	5'-GcG Gat cc Gct ata aa atG-3'	694 bp
	VP4 (694-675) (RP)*	5'-cGa att cct Gaa ttG GtG Gc-3'	
VP6	VP6 (440-459) (FP)	5'-GGa att tca aaa tGG aaG a-3'	493 bp
	VP6 (932-915) (RP)	5'-Gct GGt Gtc ata tt GGT -3'	
VP7	VP7 (1-20) (FP)	5'-atc act acc cat tgc tag cc-3'	628 bp
	VP7 (628-609) (RP)	5'-ggc att aaa atc agt aat tycg-3'	

\*FP: Forward Primer; RP: Reverse Primer

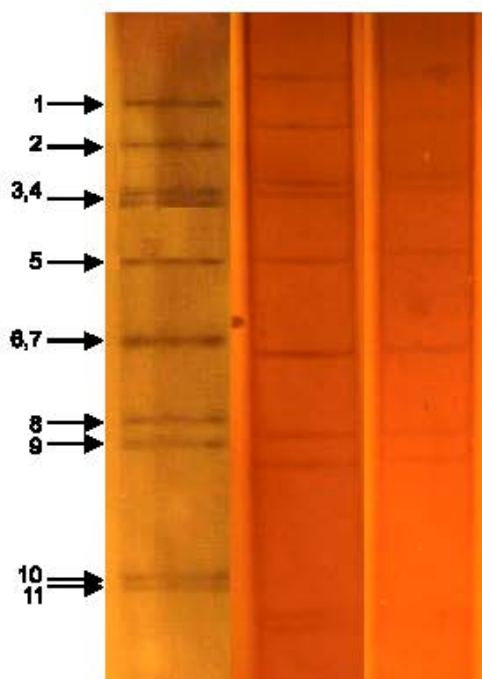


Fig. 1: Electrophoretic migration pattern of avian rotavirus. Arrow marks: Genome segments of avian rotavirus

PAGE analysis of these samples it was found that segments 3 and 4 migrated closely, segment 6 and 7 were co-migrating, segments 10 and 11 were also migrated closely whereas segment 5 was distantly migrated. All the positive samples were of short electropherotype, where segments 10<sup>th</sup> and 11<sup>th</sup> migrated very closely as compared to long electropherotype in which segments 10 and 11 migrate distantly. This is in support with results obtained by Savita *et al.* (2008).

Avian rotaviruses have been isolated from diarrhoeic chickens, turkey poulters and other avian species in various parts of the world (Yashon and Schat, 1985; McNulty *et al.*, 1984). Earlier Wani *et al.* (2003) detected mammalian like group A rotavirus with a migration pattern of 4:2:3:2 in diarrhoeic chicken from Kashmir. Minakshi *et al.* (2004) reported detection of Avian group A rotavirus having 5:1:3:2 migration pattern from Haryana in Northern India. Rotavirus strains isolated from different species can be distinguished by their electrophoretic migration patterns by RNA-PAGE (McNulty *et al.*, 1980; Todd and McNulty, 1986). These patterns can be used as specific markers of different virus population and to study the genomic diversity among rotaviruses. Surprisingly, in our study none of the electropherotype from poultry birds showed mammalian like group A rotavirus RNA migration pattern. The findings of above study are in accordance with a report

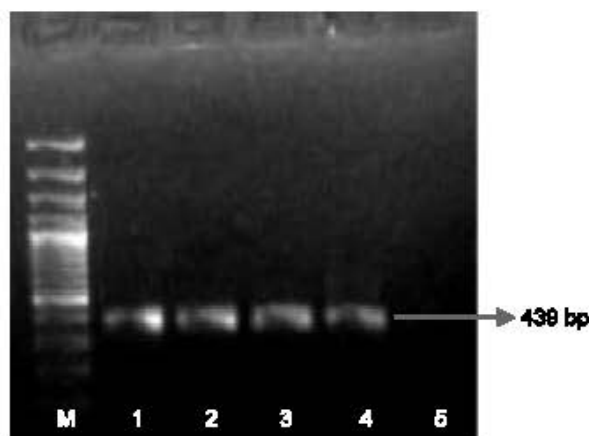


Fig. 2: Partial length amplification of VP6 gene of avian rotavirus isolates yielding 493 bp product (Lane M) 100 bp DNA ladder (1-4) Avian Rotavirus field isolates (5) Water control

of Savita *et al.* (2008) in which group D avian rotaviruses were detected in diarrhoeic faecal and environmental samples in Central India. We describe detection of avian group D rotaviruses among 60-65 weeks age poultry birds. This is in concurrence to earlier studies in India (Wani *et al.*, 2003; Minakshi *et al.*, 2004). However Ahmed and Ahmed (2006) and Legrottaglie *et al.* (1997) reported detection of avian rotavirus in broiler chickens of 1-2 weeks age early age which is contrasting to the present. All four RNA-PAGE positive samples of avian rotavirus were subjected to VP4 and VP7 gene based RT-PCR amplification using gene specific primers. After analysis of results it was found that no amplification was observed in all the four positive samples. Similarly, Deswal (2006) could not find even single PAGE positive samples of avian rotavirus showing amplification for VP4 and VP7 gene also which he described may be due to emergence of new strain. Further positive samples were subjected to partial length amplification of VP6 gene using forward and reverse primers. All four positive samples yielded a specific product of 493 bp as observed in 1% agarose gel (Fig. 2). However, no amplification was observed in water control using the same set of amplification conditions indicating the specificity of the primers. Similar findings were recorded in earlier studies by Deswal (2006). The RT-PCR offers many advantages besides high sensitivity and specificity in detection of rotavirus in faecal samples (Kang *et al.*, 2004; Fedorova *et al.*, 2005). It helps in the detection of viral nucleic acid during initial stages of infection without waiting for higher virus titer and development of immune response in the affected host species. Detection of rotavirus infection in reservoir animals and symptom less carriers is another advantage of RT-PCR.

**Conclusion:** The present study showed the prevalence of avian rotavirus among layer poultry birds. With the help of electrophoretic migration pattern of dsRNA, individual virus strain can be characterized which could be used in epidemiological investigation. The results indicate that RT-PCR based on VP6 gene can be employed as a sensitive and specific assay for rapid detection of group D avian rotaviruses in faecal samples. The present study describes first ever detection of group D avian rotavirus in Western India. With this and earlier findings it is to be noted that avian rotavirus could be considered as one of the cause of diarrhoea in poultry birds irrespective of their age group. The nucleic acid based genotypic classification of avian rotaviruses would be helpful to study genomic diversity among poultry birds of various age groups for development of rapid and sensitive diagnostic tools.

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