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# Research Article

## Distribution Frequency of Avian Rotaviruses in India, 2011–2013

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ABSTRACT

The present epidemiologic study was attempted to discern the existing status of avian rotavirus prevailing in different states of India. The frequency and dissemination of avian group A and D rotaviruses in northern and southern states of India was ascertained by using polyacrylamide gel electrophoresis with silver staining (PAGE–ss) and RT–PCR assays. A total of 215 faecal/intestinal contents of chickens (1–8 weeks old) collected during July 2011 to January 2013 were examined for the presence of rotaviruses. Of the 215 diarrheic faecal samples, 7 (3.25%) were detected positive in PAGE–ss, while 25 (11.6%) revealed amplicons of expected size in RT–PCR for group A rotaviruses. Three of the cases were positive for both group A and D rotaviruses in RT–PCR. In RNA–PAGE, rotavirus positive samples showed typical mammalian group A specific observable 4:2:3:2 genomic pattern. Additional studies are essential to corroborate the notable surveillance of rotaviruses in avian species.



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### INTRODUCTION

Rotaviruses have been documented as one of the main etiological agents of gastroenteritis in mammals and birds (Savita et al., 2008 a, b; Jindal et al., 2012; Malik et al., 2012, 2013). The virus constitutes a genome made up of 11 segments of double stranded RNA (Estes and Kapikian, 2007). The group specific VP6 protein possessing specific antigenic determinants categorize the virus into seven different groups (A to G) (Attoui et al., 2012). Among different groups, Group A rotaviruses affects both mammals and birds, while type D, F, and G have been reported only from the birds. Of these, group A rotaviruses are found to be the major cause of viral gastroenteritis in all the species of animals (Estes and Kapikian, 2007; Dhama et al., 2009; Kusumakar et al., 2010; Malik et al., 2012, 2013). The virus has been isolated from a wide variety of avian species, including turkeys, chickens, and pheasants (Gough et al., 1985,1986; Reynolds et al., 1987a,b; Theil et al., 1986; McNulty, 2003) causing Runting and Stunting syndrome (RSS) in chicken (Otto et al., 2006) and Poult enteritis syndrome (PES) in turkeys (Jindal et al., 2009; 2010). Rotavirus infections in poultry may induce subclinical manifestations, or may be connected with enteritis, dehydration, anorexia, low weight gain, and increased mortality (McNulty, 2003; Tamehiro et al., 2003). Symptoms of rotavirus infection may vary from a mild disease in young chickens to a more severe manifestation in 12 to 21–day–old chickens, characterized by unrest, litter ingestion, watery faeces, wet litter, and severe diarrhea (Barnes, 1997). The mixed aetiology of diarrhea due to avian rotavirus, *Escherichia coli* and *Salmonella* has also been reported (Savita et al., 2008a).

Reports describing the presence of group A avian rotaviruses in northern and central part of India are limited (Wani et al., 2003; Minakshi et al., 2004; Savita et al., 2008b, Niture et al., 2011). Recently, few reports came forward linking the poultry enteritis with group D avian rotaviruses across the world (Ahmed and Ahmed, 2006, Otto et al., 2012), but India far lags behind in this regard (Savita et al., 2008b, Niture et al., 2010). The present study was designed to know the distribution frequency of avian rotaviruses in northern and southern states of India using RNA–polyacrylamide gel electrophoresis (RNA–PAGE) and reverse–transcription–polymerase chain reaction (RT– PCR) detection systems.

### MATERIALS AND METHODS

#### *Clinical Sample Collection and Processing*

During the period of July, 2011 to January, 2013, intestinal contents from 1 – 8 weeks old diarrheic and post mortem enteritis cases of chickens (n=215) were collected from northern (Uttar Pradesh, Haryana, Uttarakhand) and southern (Kerala, Tamil Nadu) states of India (Table 1). Intestinal contents from chickens of same flock and same farm collected aseptically at the same time were pooled and transported on ice to the Enteric Virus Laboratory, Indian Veterinary Research Institute, Mukteswar, India. The enteric contents were processed as 10% suspensions in phosphate buffer saline (0.01 M pH 7.2), and clarified at 12,000 x g for 30 min at 4ºC to get clear supernatant containing the virus, which was archived at –80ºC until further use.



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Table 1: PAGE and RT–PCR based detection of avian rotaviruses in intestinal contents/diarrheic samples collected from chickens of different regions of India

| S. No. | States | Diarrheic intestinal contents /faecal samples tested | Samples tested positive by | |
|---|---|---|---|---|
| | | | PAGE (%) | PCR (%) |
| 1. | Uttarakhand (Pantnagar) | 142 | 4.0 (2.8%) | 16.0 (11.26%) |
| 2. | Uttar Pradesh (Bareilly) | 23 | 0.0 | 0.0 |
| 3. | Haryana (Hisar) | 10 | 0.0 | 6.0 (60%) |
| 4. | Kerala (Mannuthy) | 25 | 3.0 (12%) | 3.0 (12%) |
| 5. | Tamil Nadu (Namakkal) | 15 | 0.0 | 0.0 |
| | **Total** | **215** | 7.0 (3.25%) | 25.0 (11.62%) |

***Viral RNA Extraction and RNA–Page***

Extraction of viral RNA, polyacrylamide gel electrophoresis (PAGE) and silver staining was performed as described in our previous studies (Malik et al., 2012).

***Two Step RT–PCR Assay***

For detection and confirmation of the group A and D avian rotaviruses, two–step RT–PCR assay was executed. In the first step, reverse–transcription for cDNA synthesis from viral RNA was performed using 1.0 µl (100 ng/µl) random hexamer (Fermentas), 100 ng of viral RNA, and 2 µL of dimethyl sulphoxide (DMSO) were added to PCR tube containing sufficient volume of nuclease free water (NFW), followed by incubation of the reaction mixture at 70°C for 5 min to melt secondary structures within the template. The mixture was immediately snap chilled on ice followed by the addition of 4 µL of 5X RT buffer, 2 µL of 10 mM dNTPs (Fermentas, Lithuania), 40 U RNase Inhibitor (Ambion, USA), and 200 U MMLV–RT (Promega) and kept at 37°C for 90 minutes. The enzyme was denatured at $80^0$C for 3 min at the end of the incubation step to inactivate residual moloney murine leukemia virus–reverse transcriptase (MMLV–RT). The cDNA thus obtained was used for PCR and the remaining was kept at –20ºC until further use.

In the second step, for the detection of group A avian rotavirus, VP6 gene based PCR was implemented using the newly designed primers i.e. (sense 5´TTTGATCACTAAYTATTCACC 3´ and anti–sense 5´GGTCACATCCTCTCACTA3´) with the optimized reaction conditions (initial denaturation at 95°C for 5 min, cyclic denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 30 sec for repeated 35 cycles and a final extension temperature–time combination of 72°C for 10 min). For the detection of group D avian rotaviruses, separate VP6 gene based diagnostic primers were used (sequences not shown) with the same amplification conditions described for group A rotaviruses. The specific PCR amplicons of 227 bp for group A and 185 bp for group D rotaviruses were visualized in ethidium bromide stained 2% agarose gel and documented using Transilluminator–UV®300 (UVP Inc., Upland, USA).

## RESULTS AND DISCUSSION

The gastrointestinal infections associated with viral aetiology which occur in chickens tend to preponderate more the young birds. In the field conditions, these infections are convoluted by other infectious agent's viz. bacteria or virus, making it problematic to measure the factual role of these agents in naturally befalling gastrointestinal diseases (Savita et al., 2008a). Thus, an understanding of the viruses that cause gastrointestinal tract disease in poultry, along with an understanding of their epidemiology is necessary for development of be–fitting rheostat measures. Initial works for classification of rotaviruses in to different groups (A–G types) was based on migration patterns of RNA segments in the PAGE (Pedley et al., 1986; Estes and Kapikan, 2007)

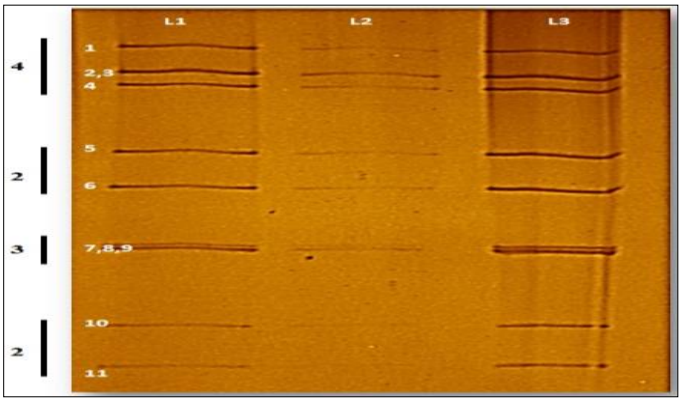


**Figure 1: Mammalian–like genomic segments migration pattern (4:2:3:2) of avian group A rotaviruses in silver stained RNA–PAGE.** RNA segments are numbered according to the electrophoretic mobility in polyacrylamide gel. Lanes 1, 2, and 3 represents group A rotavirus from Uttarakhand, Haryana and Kerala, respectively.

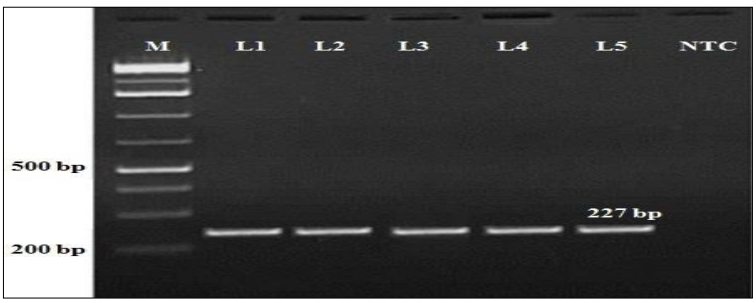


**Figure 2: RT–PCR amplification of group specific partial VP6 gene of avian rotavirus.** Lane M: 1Kb plus DNA ladder (Fermentas); Lanes 1–5: Group A rotavirus (227 bp); NTC–no template control. All amplicons were visualized in 2% ethidium bromide stained agarose gel.


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In this study, initial screening by RNA–PAGE for the presence of rotaviruses revealed typical genome segments migration pattern of 4:2:3:2 (Fig. 1) explicit of mammalian group A rotaviruses in 3.25% cases (7/215). Avian rotavirus with an electropherotype similar to described here for group A rotaviruses was also detected in 4% (3/75) of diarrheic adult chickens for the first time in India (Wani et al., 2003). Interestingly, in contrast to avian group A rotavirus migration pattern of 5:1:3:2 (Schumann et al., 2009), migration of segment 5 close to 6 and triplet of segments 7, 8 and 9 in all the positive samples clearly indicated mammalian group A rotaviruses. Since, the first detection of avian group A rotaviruses a decade ago in India, several other reports successively recorded presence of rotavirus infection in the range of 7.84% to 22.2% (Wani et al., 2003; Minakshi et al., 2004; Savita et al., 2008b; Niture et al., 2011), suggestive of consistent commonness of rotavirus infection in the birds. In addition to more common group A avian rotaviruses, group D avian rotaviruses from central (77.7%) and western (7.84%) region of India have also been testified (Savita et al., 2008b; Niture et al., 2010).

The RT–PCR assay, being the much more sensitive, detected 11.6% samples (25/215) positive for group A rotavirus (Fig. 2) and mixed infection of both group A and D rotaviruses in 1.4% (3/215) samples. Our results are in compliance with the earlier findings where higher prevalence of group A compared to group D avian rotaviruses was recorded (Wani et al., 2003; Minakshi et al., 2004). At the same, in one of our previous study, the prevalence rate of group A rotaviruses was less (22.2%) compared to group D rotaviruses (77.8%) in the chicken flock of central India (Savita et al., 2008b). In another report from Maharashtra, Niture et al. (2010) also showed higher prevalence rate of group D than group A avian rotavirus.

To the best of our knowledge, report of co–infection with different types of avian rotavirus in chickens are lacking so far. The three samples showing mixed infection of group A and D rotaviruses in RT–PCR assay did not show any positive presence of rotavirus dsRNA in conventional RNA–PAGE, which could be due to lower copy number of the group D rotavirus particles which failed its detection due to lower sensitivity limit of RNA–PAGE. The regional distribution of avian rotaviruses disclosed the high prevalence in Uttarakhand (7.44%, 16/215) followed by Haryana (2.8%, 6/215) and Kerala (1.3%, 3/215). None of the sample from Uttar Pradesh and Tamil Nadu yielded positive result, but before concluding absence of avian rotavirus in these regions, extensive epidemiological studies on more number of enteritis/diarrheic samples needs to be assessed.

In conclusion, the present study reports the occurrence of mammalian type group A avian rotaviruses from the southern and northern states of India using PAGE and RT–PCR assays. The existence of more than one virus group within a single host may not only complicate the severity of enteritis cases in poultry flocks but also could lead to emergence of reassortants having more pathogenic potential. Further studies will be essential to understand the evolving epidemiology of rotaviruses in avian species

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