DETECTION OF AVIAN ROTAVIRUS USING THE REVERSE TRANSCRIPTION (RT)-PCR AND RNA-PAGE IN DIARRHOEIC POULTRY FROM KERALA, INDIA

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Abstract

Rotavirus is one of the most common viral causes of diarrhoea in poultry causing significant economic losses. Hence a study was undertaken to detect the presence of rotavirus in faecal samples by RT-PCR and the RNA profile of the virus was analysed by PAGE for subsequent grouping. One hundred and forty three faecal samples of poultry having diarrhoea were collected from University poultry and duck farm, Kerala Veterinary and Animal Sciences University, Mannuthy and various regional poultry farms in Kerala. All the samples were screened for the presence of avian rotavirus by RT-PCR and RNA-PAGE. Among the 143 samples, 5 (3.49 per cent) were found to be positive by RNA-PAGE. The 11 segments of the rota viral genome was observed and showed a migration pattern of 4:2:3:2 which was typical for mammalian like group A rotavirus. Eleven samples (7.69 per cent) were detected positive in RT-PCR and was found to be more sensitive in detecting avian rotavirus than RNA-PAGE.

Key Words: *Avian rotavirus; RNA-PAGE; Silver staining; RT-PCR.*

Rotavirus first isolated in 1968, is now recognized as the most important viral agent causing neonatal diarrhoea (Doyle and Hutchings; 1946Mebus *et al.*, 1969).

The rotavirus infection has been reported from many countries world-wide including India. The virus affects the younger age groups, and the disease has to be differentially diagnosed from other enteropathogenic viral and bacterial infections. Since these viruses are highly contagious, a rapid and specific diagnosis is absolutely necessary to control the spread of the disease. The conventional methods employed for the detection of avian rotavirus like electron microscopy and virus isolation are considered to be relatively expensive, laborious and time consuming. Recently, a nucleic acid based technique like Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) has been introduced for the rapid detection of avian rotavirus from faecal samples. Ribonucleic acid-Poly Acrylamide Gel Electrophoresis (RNA-PAGE) differentiates avian rotavirus groups based on electrophoretic RNA migration. Hence the present study was

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undertaken to detect the presence of avian rotavirus in poultry by RT-PCR and RNA-PAGE.

Material and Methods

Collection of samples

A total of 143 faecal samples were collected from poultry having diarrhoea (Table 1). Collection of samples was made using dry sterile cotton swab and after collection, the swabs were immediately immersed in RNA later and preserved at -50º C until use.

Extraction of RNA

The RNA was extracted from the faecal samples as per the manufacturer's protocol, Sigma-Aldrich Inc. USA with minor modifications. All the micro tips and microcentrifuge tubes used in the process were washed with DEPC treated water to make it RNase free and were sterilized by double autoclaving.

RNA-PAGE

The rotavirus genome was detected in faecal samples by RNA-PAGE following the method of Ambily (2007) with minor modifications.

Reverse Transcriptase-Polymerase Chain Reaction

The primers used for the amplification of VP6 gene of avian rotavirus (Accession No. DQ096805.1) was designed using the NCBI primer-BLAST software. The sequences used in the study were

Forward primer (F1) : 5'- TATGACACCAGCCGTAGCAG-3'

Reverse primer (R1) : 5'- GTTGGACCAGTATTTCC -3'

The RT-PCR was carried out as per the method of Ambily (2007) with minor modifications. The first strand cDNA was synthesized from viral RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). The following reagents were added into sterile, nuclease-free tube in the indicated order: Viral RNA (8µL), Forward (or) Reverse primer of rotavirus (1µL) and Nuclease free water (added upto 12µL). The tube was then placed in thermal cycler at 90ºC for five min and snap chilled on ice. Then 5X Reaction buffers (4µL), Ribolock RNase Inhibitor (1µL), 10mM dNTP Mix (2µL) and RevertAid M-MuLV Reverse Transcriptase (1µL) were added. The reaction mixture was incubated at 25ºC for five minutes. The reverse transcription was carried out at 42ºC for 60 min in thermal cycler. This was followed by heat inactivation of M-MuLV RT at 70ºC for five minutes. The strand of cDNA thus synthesized was used as template for PCR. Human rotavirus vaccine (RotaTeq- which was live, oral and pentavalent) was used as the positive control.

The PCR reaction mixture (25µL) was prepared by mixing cDNA (5µL), F1 of rotavirus (1µL), R1 of rotavirus (1µL), 10X PCR buffer (2.5µL), 10mM dNTPs (0.5µL), *Taq* polymerase (5 U/µL) 0.5µl and nuclease free water (12.5µL) on ice. The amplification were carried out in thermocycler with an initial denaturation at 95ºC for five minutes, followed by the sequence of 35 cycles as follows: 95ºC for 25 sec, 50.4ºC for 15 sec. and 72ºC for 2 min. 50 sec.. This was followed by final extension at 72ºC for eight minutes. The PCR product was detected by electrophoresis at 50 V and 16 mA in one per cent agarose gel in Tris Acetate EDTA buffer (1X).

Results and Discussion

In the present study, the RNA concentration and purity was measured by spectrophotometer (NanoDrop 2000C). Similar method of RNA extraction of rotavirus from diarrhoeal faecal samples from bovines using TRIzol was employed by Ambily *et al.* (2009) and they got good quality RNA.

By RT-PCR the target gene was successfully amplified and generated an amplicon of 188 bp similar to positive control (Fig. 1), thus confirming the sensitivity of the primers. In addition, negative control, comprising of nuclease free water and PCR reagents and known negative samples when

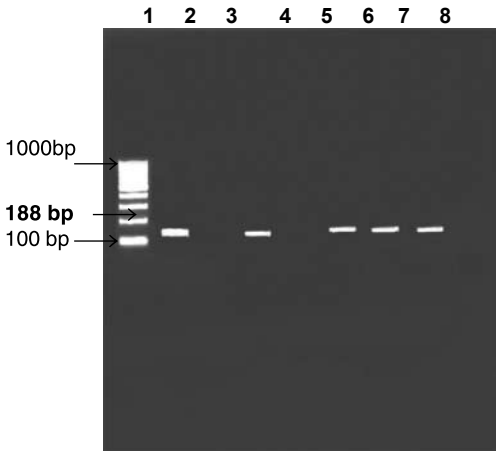

Fig. 1. Agarose gel electrophoresis of RT-PCR amplified product of Avian rotavirus Representation). Lane 1 - 100 bp ladder, Lane 2- Positive Control, Lane 3- Negative Control, Lane 4, 6, 7 and 8- Positive Samples, Lane 5- Negative Sample

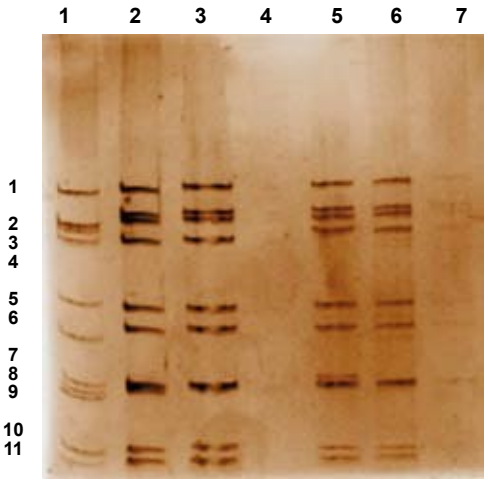

Fig. 2. RNA Profile of Avian rotavirus (Representation). Lane 1- Positive Control, Lane 2,3,5,6,7 - Positive Samples, Lane 4- Negative Sample

subjected to RT-PCR failed to yield any amplicons and thus proved the specificity of the primers. It is presumed that the quantity of PCR product is directly proportional to its template DNA concentration. In present study also, it was noticed that the thickness of the amplicons generated from positive control *i.e.,* the vaccine was considerably more than that of the positive samples, indicating low concentration of the virus in the clinical samples. Among 143 samples tested, 11 (7.69 per cent) were found positive for avian rotavirus by RT-PCR.

In RNA-PAGE followed by silver staining, 5 out of 143 samples (3.49 per cent) were found to be positive for avian rotavirus and revealed a 4:2:3:2 migration pattern specific for mammalian like Group A rotavirus. The positive control also showed similar results (Fig. 2).

The faecal samples collected from poultry having diarrhea during the study were subjected to RNA-PAGE and RT-PCR. Similar investigation strategy was adopted by Arguelles *et al.* (2000) and Ambily *et al.* (2009). It was observed that, five (3.49 per cent) and eleven (7.69 per cent) samples were found to be positive by RNA-PAGE and RT-PCR, respectively (Table 2). Six of the samples that yielded negative results in RNA-PAGE turned positive in RT-PCR, and hence RT-PCR could be proved as a more accurate and sensitive method for the detection of avian rotavirus infection from faecal samples (Table 3), which

Table 1. Results of RNA-PAGE

Sources of Samples	No. of samples screened by RNA-PAGE	No. of positive samples	Per cent positivity
University Poultry and Duck farm, Mannuthy	62*	2**	3.22
RPF, Athavanad	9	0	0
RPF, Malampuzha	10	0	0
RPF, Chathamangalam	20	2	10
RPF, Mundayad	20	0	0
ILFC, Pookode	20	1	5
Clinical sample, University Veterinary Hospital, Mannuthy	2	0	0
Total	143	5	3.49

* Comprises 35 (Chickens); 9 (Quail); 8 (Turkey) and 10 (ducks); ** Chicken Samples

Table 2. Comparison between RNA-PAGE and RT-PCR

Test	No. of samples tested	No. of positive samples	Per cent positivity
RNA-PAGE	143	5	3.5
RT-PCR	143	11	7.7

Table 3. Association between RT-PCR and RNA-PAGE

	RNA-PAGE			
RT-PCR		+	-	Total
	+	5(a)	6(b)	11(a+b)
	-	0(c)	132(d)	132(c+d)
	Total	5(a+c)	138(b+d)	143(a+b+c+d)

Sensitivity = a/ (a+c) = 100%; Specificity = d/ (b+d) = 95.65%; Accuracy =a+d/(a+b+c+d) = 95.80%

is in perfect agreement with the observations of above mentioned researches.

To conclude, the study confirmed the prevalence of avian rotavirus infection in diarrhoeic poultry in Kerala with RT-PCR and RNA-PAGE. Reverse Transcriptase-Polymerase Chain Reaction could detect 7.69 per cent positive samples and was found to be a more sensitive assay than RNA-PAGE.

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