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Molecular Survey of Enteric Viruses Associated with Poultry Enteritis in Southern Rajasthan, India

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Abstract

Enteric viruses play major role in affecting the poultry industry worldwide, the occurrence of enteric viruses in poultry is still very poorly explored in India. Therefore, a molecular survey has been performed to determine the presence of enteric viruses among enteritis affected poultry flocks of Udaipur and Chittorgarh districts of Southern Rajasthan, India. Total 60 pooled samples of intestine were collected from enteritis affected dead birds, DNA and RNA viruses were detected by PCR and reverse transcriptase PCR respectively. Enteric viruses namely Fowl adenovirus-I(FADV-I), Chicken parvovirus (CPV), Avian coronavirus (ACV), and Chicken astroviruses (CAstV) were detected in 73.32 % of investigated samples. FAdV-I (21.66%) was detected as highest single virus infection followed by ACV (13.33%). Combinations of two or more enteric viruses were simultaneously identified in 38.33% samples.

Keywords: *Avian coronavirus*, *Chicken parvovirus*, *Chicken astrovirus*, Enteric Viruses, *Fowl adenovirus*, Poultry Enteritis

Introduction

Enteritis is one of most important multi-factorial diseases of the poultry industry, responsible for heavy economic losses, as it decreases feed absorption, resulting in growth retardation, impaired feed efficiency, immunosuppression and sometimes increased mortality due to secondary invaders (Koo *et al.,* 2013). Further any change in delicate and balanced microenvironment *i.e.,* microflora of intestine leads to poor vitamin production immune-suppression and increase growth of harmful bacteria in the intestine of chickens (Yegani and Korver, 2008). The viruses commonly associated with poultry enteritis are: *Fowl adenovirus-I* (FAdV-I), *Chicken parvovirus* (ChPV), *Avian coronavirus* (ACV), *Chicken astroviruses* (CAstV) and *Avian nephritis virus* (ANV). *Avian Reovirus* (AReo) and *Avian Rotavirus* (ARtV) (Zsak *et al*., 2012; and De la Torre *et al.,* 2018). Enteric viruses are mainly responsible for primary damage to the host tissue which provides opportunity to secondary agent like bacteria (*Salmonella*, *Escherichia coli and Clostridium etc.)* or parasite (*Eimeria spp.*) to invade gastrointestinal tract (GIT) tissue, leading to severe irreversible injury and appearance of clinical signs (Kaithal *et al*., 2016).

Geo-climatic conditions of different areas play an important role in prevalence of any disease. So, the area specific prevalence study always gives an insight on occurrence of the disease and their etiological agents in particular area. There are many reports of enteric viruses from different countries such as United States, U.K., Germany, Japan, Iraq, Canada, Australia, China, Brazil, Korea *etc.* (Decaesstecker *et al*., 1988; Hess 2000; Hewson *et al*., 2010; Jindal 2010a; Mahmood *et al*., 2011; Roussan *et al.,* 2012; Koo *et al*., 2013; and Niu *et al.,* 2018). However scanty data about prevalence of enteric viruses are available from India (Kaithal *et al*., 2016). So, a molecular survey was performed for a broad range of enteric viruses, namely FAdV-I, ChPV, CAstV, ANV, and ACV. To our best of knowledge, this is the primary molecular survey of RNA and DNA viruses associated with poultry enteritis from Rajasthan, India.

Materials and Methods

This study was conducted in Department of Veterinary Microbiology, CVAS, Navania, Vallabhnagar, Udaipur, (RAJUVAS, Bikaner) Rajasthan, India, for a period of January 2018 to March 2019.

Ethical Approval

The samples for the study were collected during the post mortem of poultry and there is no involvement of live birds or animal during the study. So, the ethical committee approval is not required.

Chicken Flocks

Between 2018 and 2019, five commercial and 25 backyard poultry farms from two districts of Southern Rajasthan were selected for molecular examination of the following enteric viruses namely FAdV-I, ChPV, CAstV, ANV, and ACV. Birds ranging from hatching to 4 weeks old died due to enteritis and had a history of growth retardation were brought to Department of Veterinary Microbiology CVAS, Navania, Udaipur, Rajasthan.

Postmortem Examination and Sample Collection

The poultry birds were subjected to postmortem examination, all visceral organs were examined for gross lesions (Fig. 1). Intestine part showing hemorrhages, dilatation with thinning of wall and containing undigested food contents were collected aseptically.

Sample Processing and Viral Nucleic Acid Extraction

Intestinal samples collected from four birds of same age group were pooled together, was mechanically macerated with sterile sand in mortar and pestle and diluted with 10 volume of PBS, and processed as described previously (De la Torre *et al.,* 2018). Viral DNA/RNA was extracted from 250 µl of the intestinal sample supernatant using Gene JET Viral DNA/ RNA Purification Kit (Thermo Scientific) according to manufacturer's instruction.

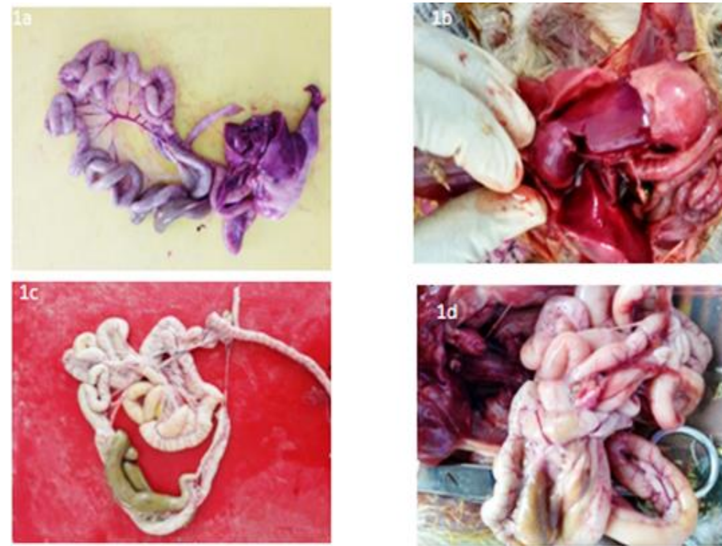

Figure 1: Postmortem examination of poultry; **1a and1b:** Fifteen- and sixteen-days old chick intestine showing severe hemorrhagic lesions. **1c:** Twenty-four days old poultry intestine, thin walled with distended ceaca and watery contents in it. **1d**: Twenty-eight days old poultry intestine distended, pale and hemorrhagic lesions.

PCR for *Fowl adenovirus* and *Chicken parvovirus*

FAdV and ChPV were detected by PCR targeting Hexon gene of FAdV and nonstructural (NS) gene of ChPV. Briefly 20 µl reaction mixtures contained forward and reverse primers specific for the target gene 0.5 µl each (10pico-mol/ µl), DNA template 2 µl, 2X PCR master mix (Thermo scientific) 10 µl and nuclease free water (NFW) 12µl. Thermal conditions for amplifying the ChPV gene were as follows; one cycle for hot start at 95^{0}C for 5 min, followed by 35 cycles of amplification, (95^{0}C for 30 second (sec), 55^{0}C for 50 sec, 72^{0}C for 50 sec), and one cycle of final extension at 72^{0}C for 10 min. Thermal cycle conditions for amplification of FAdV-I gene were the same as above except annealing temperature was kept at 51^{0}C. Cycling reactions were conducted using a PCR thermal cycler (BIO RAD, T100). PCR product were visualized by electrophoresis using 1.5% agarose gel running at 70 volts and band of amplicon size 897bp (FAdV) and 1217bp (ChPV) was visualized in trans-illuminator (Fig. 2). Details of all primers used in this study are summarized in Table 1.

Figure 2: Gel images of PCR amplicons of Hexon gene of FAdV-1(2a), NS gene of ChPV(2b) and ORF 1 b gene of CAstV (2c)

Reverse Transcriptase Reaction

The RNA extracted was subjected to a reverse transcription reaction to obtain complementary DNA (cDNA). Ten µl RNA template was taken in a 200 µl PCR tube and 0.5 µl Random hexamer was added and incubated at 72^{0}C for 7 min, followed by immediate chilling on ice. The reaction mixture was centrifuged briefly and 4 µl 5X RT buffer, 1µl 10 mM dNTPs, 0.5 µl 40U/µl RNAse inhibitors, 0.5µl 200U/µl Revert Aid Reverse Transcriptase (Thermo scientific) and 3.5 µl NFW were added. A reverse transcription reaction was performed under the following conditions 25^{0}C for 10 minutes, 45^{0}C for 45 min. and 94^{0}C for 10 min. in thermal cycler. cDNA obtained was

subjected to PCR.

Table 1: Detail information about the primers used in PCR and RTPCR

Virus	Gene Target	Primer	Nucleotide sequences (5'-3')	Amplicon (bp)	Annealing temperature	Reference
FAdV-I	Hexon	Forward	5'-CAARTTCAGRCAGACGGT-3'	897	51^0C	Meulemans *et al.,* 2001
		Reverse	5'-TAGTGATGMCGSGACATCAT-3'			
ChPV	NS PVF1	NS1-F	5'-GATTCAGGCGAATTCGTGGC-3'	1217	55^0C	Procured from NCVTC, Hisar
		NS1-R +	5'- ACGTGTTCTTCCGTCATGCT -3'			
ACV	UTR	UTR-11 (Forward)	5'-GCTCTAACTCTATACTAGCCTA-3'	179	48^0C	Cavanagh *et al.,* 2002
		UTR-31 (Forward)	5'-GGGCGTCCAAGTGCTGTACCC-3'			
		UTR-41 (Reverse)	5'-ATGTCTATCGCCAGGGAAATGTC-3'			
CAstV	ORF 1b (pol)	Forward	5'-GAYCARCGAATGCGRAGRTTG-3'	362	51^0C	Michael Day *et al.,* 2007
		Reverse	5'-TCAGTGGAAGTGGGKARTCTAC-3'			
ANV	ORF 1b (pol)	Forward	5'-GYTGGGCGCYTCYTTTGAYACCRT-3'	473	50^0C	Michael Day *et al.,* 2007
		Reverse	5'-CRTTTGCCCKRTARTCTTTRTGAY-3'			

RT-PCR for *Chicken astrovirus* and *Avian nephritisvirus*

PCR reaction was carried out in a total volume of 20 µl containing 2µl c-DNA as template, 0.5 µl of each forward and reverse primer (10pmol/µl), 10 µl of 2 x PCR master mixes (Thermo scientific) and 7µl NFW. The thermal conditions were as follows; a cycle of 95^0C for 5 min., 35 cycles of amplification (at 95^0C for 30 sec, 50^0C for 30 sec, and 72^0C for 50 sec), and final extension was done at 72^0C for 10 min. To amplify the genes from AstV and ANV viruses the primer sets CAS pol1F and 1R and ANV pol1Fand R were used respectively. PCR product were visualized by electrophoresis using 1.5% agarose gel running at 70 volts and band of amplicon size 362bp (AstV) and 473 bp (ANV) was visualized in trans-illuminator (Fig. 2).

RT -PCR for *Avian coronavirus*

First, we performed PCR from cDNA template targeting ACV UTR gene (Cavanagh *et al.,* 2002) Table 1. The procedure, volume and cyclical condition were kept same as described previously (De la Torre *et al.,* 2018). Further second amplification of hemi-nested PCR was carried out using one µl the amplified product of primary amplification as template, with forward and reverse primers (UTR-31and UTR-41), rest reagents and thermal conditions were kept same as primary reaction. PCR product were visualized by electrophoresis using 1.5% agarose gel running at 70 volts and band of amplicon size 266bp and 179 bp was visualized on uv trans-illuminator (Fig. 3).

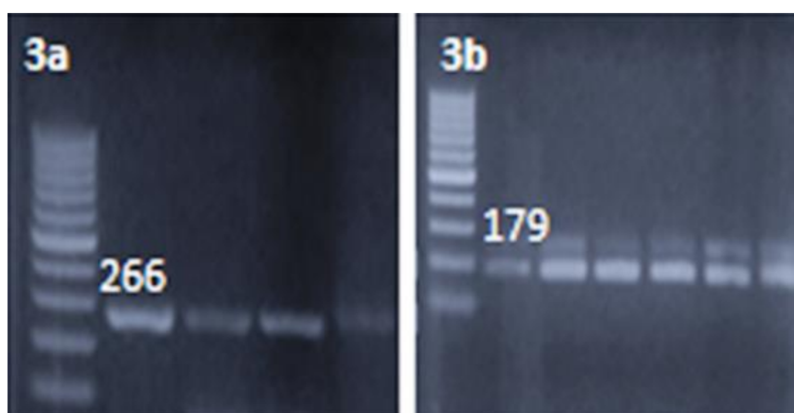

Figure 3: Gel images of Primary amplicon of hemi-nested PCR of UTR gene of ACV (3a), secondary amplicon product of ACV UTR gene (3b)

Results and Discussion

Occurrence of Enteric Viruses

Enteric viruses were identified in 73.32% (44/60) of pooled samples from commercial and backyard chicken flocks of two districts of southern Rajasthan. The breakdown of enteric viruses was as follows FAdV-I 51.66%, ChPV10%, ACV 50%, CAstV3.33%and ANV0%. Two or more viruses were identified in 38.3% (23/60) of tested samples *i.e.* FAdV-1 + ACV (25%), ChPV + ACV (6.66%), FAdV-1 + CAstV (1.6%), CAstV + ACV (1.6%), FAdV-1+ ChPV +ACV (3.48%) (Table 2).

In present report high proportion (73.33%) of samples were found positive for single or combination of enteric viruses, which indicates that enteric viruses are widely prevalent in enteritis affecting poultry flocks in the study area and these viruses may play an important role in causation of enteritis. Up to our best knowledge present study is a primary report from Rajasthan, India investigating the prevalence of both DNA and RNA enteric viruses from the poultry enteritis. A higher prevalence of 93.8% of enteric RNA viruses (CAstV, ANV, and ARV) has been reported from Haryana state of India (Kaithal *et al*., 2016). Difference in overall prevalence may be due to the geographical and climatic differences. Globally various researchers have also reported the presence of different enteric viruses from enteritis affected chickens and turkeys (Jindal *et al.,* 2010; Roussan *et al*., 2012, Moura-Alvarez *et al*., 2013 and Mettifogo *et al*., 2014) at different prevalence rate. FADV-I showed its presence in 51.66% samples, as single virus in 21.66% and as mixed with other enteric viruses in 30%. Association of FAdV-I with enteric disorders has been reported very recently by De la Torre and co-workers. They reported 18 strains of FAdV-I as a causative agent of enteritis in poultry (De la Torre *et al*., 2018).

Table 2: Occurrence of enteric viruses in intestinal contents of broiler chickens at Southern district of Rajasthan

Name of Virus	Number of Positive samples (Total pooled sample tested N=60)	Percentage Prevalence (Total pooled sample tested N=60)
Single		
FAdV-I	13	21.66%
ACV	8	13.33%
CAstV	0	0%
ChPV	0	0%
ANV	0	0%
Total (Presence of single viral infection)	21	35%
Mixed infection		
FAdV-I + ACV	15	25%
ChPV + ACV	4	6.66%
FAdV-I + CAstV	1	1.60%
CAstV + ACV	1	1.60%
FAdV-I+ ChPV + ACV	2	3.48%
Total (Presence of Mixed viral infection)	23	38.33%
Grand Total	**44**	**73.32%**

Avian corona virus (ACV) a common cause of infectious bronchitis and also known as infectious bronchitis virus (IBV); besides this, role of this virus in enteric diseases is well documented. Association of this virus with RSS has been proved by inoculation of 14 positive AIBV samples in the embryonated eggs from commercial farms, resulted in stunted growth of the embryo (El-Behery *et al*., 2016). In our study, ACV was recorded in 50% (30/60) samples. Among these 13.33% were recorded as single infection, while 36.66% were found as mixed infection with other enteric viruses. We could not find published report of occurrence of ACV virus associated with enteritis in poultry from India. Globally various workers reported higher prevalence of ACV *i.e.,* 42.9% by Mettifogo *et al*., 2014 and 58.9% by De la Torre *et al*., 2018. Marked difference in prevalence may be attributed due to change in the geographical and climatic conditions of the regions.

Chicken parvo viruses have also been detected in a high proportion from of chicken flocks suffering from enteritis; this virus can cause growth retardation bed feathering and bone disorders in broilers chickens when experimentally infected (Zsak *et al*., 2012 and koo *et al.,* 2013). In this report low prevalence of ChPV (10%) was recorded than

reported from other countries (Koo *et al.,* 2013; Mettifogo *et al*., 2014 and De la Torre *et al*., 2018) with prevalence of 26.5%, 12.1% and 6.1% respectively. We find ChPV simultaneously with other enteric viruses especially FAdV and ACV. Extent of co-infection with other pathogens in ChPV infected flocks indicates that ChPV infection may only occur in immunosuppressed hosts. However, ChPV may also causes immuno-suppression as reported (Palade *et al.,* 2011).

CAstV was detected in very low prevalence CAstV (3.33%) where as ANV not detected in any sample. However, the association of CAstV and ANV with RSS has been reported throughout the world (Pantin-Jackwood *et al*., 2008; Smyth *et al*., 2009; Koo *et al.,* 2013 and De la Torre *et al*., 2018). In India higher prevalence of CAstV was reported as 80% from Haryana (Kaithal *et al.,* 2016). Variation among different state of same country also has been reported by De la Torre and coworkers. They covered 12 states of Brazil and reported CAstV only in two states and ANV from seven states, which shows, occurrence of the virus can varies in a small geographical area (De la Torre *et al*., 2018). This variation also emphasized the importance of area wise prevalence of infectious diseases. Combination of enteric viruses has been detected in present study, were also reported earlier *i.e*., Roussan *et al*., 2012; Koo *et al*., 2013 and De la Torre *et al.,* 2018. Although presence of these enteric viruses was identified in commercial chickens, their exact role in pathogenesis of enteritis and RSS should be evaluated in future. Further the correlation of occurrence of more than one virus with the severity of the disease which needs to be explored.

Conclusion

To conclude, we find out the prevalence of enteric viruses in broilers chickens affected with enteritis at broiler farms of southern districts of Rajasthan India. Forty-four (73.33%) samples were found positive for single or combination of enteric viruses out of 60 pooled samples tested. 35% samples were found positive for presence of single virus. Whereas 38.33% pooled samples were found positive for presence mixed viral infection,five combinations were recorded as FAdV-I+ACV(25%), ChPV + ACV (6.66%), FAdV-I + CAstV (1.66%), CAstV + ACV(1.66), and FAdV-I+ ChPV + ACV (3.77%).

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Conflict of Interests

There is no conflict of interest.

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