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Detection, prevalence and molecular characterization of avian rotavirus

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ABSTRACT

Rotaviruses are the foremost cause of acute gastroenteritis. Outbreaks of rotavirus infection can lead to significant economic losses in livestock and poultry industry. Group A and D rotavirus are the predominant enteric virus groups in birds. Limited prevalence studies are available to date in India. The present study was undertaken to study the prevalence, molecular characterization and sequence analysis of avian rotavirus in and around Mathura region. Poultry samples (20 fecal samples and 80 intestinal content samples) were screened by VP6 gene based diagnostic PCR for AvRVA and AvRVD. All samples were negative for Group A rotavirus. Five fecal samples and 22 intestinal samples were positive for AvRVD-VP6 gene giving an amplicon of 185 bp and overall prevalence of 27%. Positive samples screened for VP4, VP7, NSP4 and NSP5 genes of AvRVD were negative for VP4, VP7 and NSP4, but six samples were positive for NSP5 gene giving an amplicon of 652 bp. Cloning and sequencing showed 99.4 to 100% sequence similarity among all six isolates at the nucleotide level. The per cent nucleotide similarity with chicken RVD isolates was in the range of 78.2-85.1%. All isolates showed highest similarity with chicken isolate from South Korea (85%) followed by Germany (81.5%) and Australia (78.2%). The phylogenetic analysis exhibited all six isolates clustered together in a separate clade with isolates from Australia, Germany and South Korea.

Keywords: Avian rotavirus, NSP5, Phylogenetic analysis, RVD

The infectious diseases of the gastrointestinal tract have a worldwide distribution and a variety of etiologies. Among the viral etiology, rotaviruses (RVs) are the foremost cause of acute gastroenteritis in neonates of humans. Despite ongoing vaccination programs in human children, rotavirus infections resulted in 128,500 deaths in 2016 worldwide (Patzina-Mehling *et al.* 2020). In Southeast Asia, 40.78% of all diarrheal disease in children were caused by RV infection during 2008–2018 RV surveillance (Lestari *et al.* 2020). Besides human, high prevalence of rotavirus infection has been reported in several animal species, including birds (Otto *et al*. 2012, 2015). Outbreaks of rotavirus infection can lead to significant economic losses in livestock and poultry industry (Vlasova *et al.* 2017, Castells *et al.* 2018).

Rotaviruses are members of the genus *Rotavirus* within the family Reoviridae. Rotaviruses of species A (RVA), D, F and G have been detected in several species of birds with the predominance of RVA and RVD (Dhama *et al.* 2015). Detection of virus nucleic acid or antigen in feces to genotyping by multiplex RT-PCR are well recognized epidemiological tools for examining rotavirus strain diversity followed by sequence comparison and phylogenetic analysis that add to understanding the evolution of rotavirus (Trojnar *et al.* 2013).

Avian rotavirus have been reported from various geographical regions, including France, Egypt, Argentina, Brazil, Nigeria, Bangladesh and India (Theil *et al.* 1986, Hemida 2013). It has been isolated from turkeys, chickens, pigeons, pheasants, parrots and parakeets (Dhama *et al.* 2015). The young birds (1-2 weeks) are more susceptible to rotavirus infection, suffering from diarrhea and reduced feed intake resulting in growth retardation and increased mortality, even though co-infections with other pathogens seem to play a role in disease development (Jindal *et al.* 2014). RVD first detected as virus 132 and rotavirus–like viruses (McNulty *et al.* 1981) has been reported from many places, including a report from Nigeria (Pauly *et al.* 2017). RVD was identified as one of the causes in the pathogenesis of runting and stunting syndrome having a high negative impact on the poultry sector (Otto *et al.* 2006, Gallego *et al.* 2022). Recently, rotavirus has been found to be the single causative agent of young pigeon disease syndrome with high mortality (Harzer *et al.* 2021). Although, RVD infection has been documented in poultry birds, limited prevalence studies are available to date.

Animal rotaviruses are considered as a potential threat to humans due to the possibility of the genetic reassortment.

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Reassortants containing genome segments from human and mammalian animal RVA strains have been frequently described, underlining the zoonotic nature of mammalian animal RVs (Martella *et al.* 2010, Doro *et al.* 2015). In contrast, only little is known about zoonotic transmission and reassortment between avian and mammalian RV strains (Trojnar *et al.* 2013). Thus, accumulation of the sequence information on avian rotaviruses is important to evaluate the genetic relationship between avian and mammalian rotaviruses and to study the potential genetic and antigenic variability among rotaviruses. Keeping this in view, the present study was carried out to study the prevalence, molecular characterization and sequence analysis of avian rotavirus in and around Mathura region.

MATERIALS AND METHODS

Samples: Poultry samples consisting of 20 fecal and 80 intestinal content samples were collected during September 2017 to December 2018 from organized (Instructional Poultry Farm, DUVASU, Mathura) and unorganized poultry units / meat shops in and around Mathura region. Both diarrheic and non-diarrheic samples were collected. Suspension (10% w/v) of fecal / intestinal content in phosphate buffered saline (PBS), pH 7.4 was centrifuged at 2000 g for 20 min to remove coarse particulate matter and the upper aqueous layer was filtered through 0.22 µm syringe filter, and stored at -20°C until required.

Viral RNA extraction: The viral RNA was extracted from 500 µl of 10% fecal / intestinal suspension in PBS using an equal volume of TRI reagent (Merck). RNA was eluted with Nuclease Free Water (NFW) in a final volume of 25 µl and assessed qualitatively and quantitatively using spectrophotometer (Thermo-Scientific, USA).

Preparation of complementary DNA (cDNA) by Reverse Transcription (RT): The extracted RNA was reverse transcribed using the Applied Biosystem High-Capacity cDNA Reverse Transcription Kit (ThermoFischer Scientific). Briefly, to prepare RT master mix, 2 µl 10× RT buffer, 0.8 µl 25× dNTP mix (100 mM), 2 µl 10× RT random primers, 1 µl MultiScribe Reverse Transcriptase, 4.2 µl nuclease-free water were mixed on ice into each 0.5 ml micro-centrifuge tube and an equal volume of RNA sample was added. The tubes were sealed and spun down briefly and incubated in a thermocycler at 37°C for 120 min. After incubation, reaction mixture was heated at 80°C for 3 min to denature the residual RT enzyme. The cDNA thus obtained was kept at -20°C till further use.

Primers: Forward and reverse primers were synthesized from ILS, Gurugram and GCC Biotech (I) Pvt. Ltd. (Table 1).

RT–PCR: For the detection of Group A avian rotavirus, VP6 gene based PCR was performed on 20 fecal and 80 intestinal samples using the specific primers (ARVA6-1F and ARVA6-1R) with the optimized reaction conditions (initial denaturation at 95°C for 5 min, cyclic denaturation at 94°C for 30 sec, annealing at 54°C for 1 min and extension at 72°C for 1 min for 40 cycles and a final extension temperature–time combination of 72°C for 10 min). Similarly, for Group D avian rotavirus, VP6 gene based PCR was performed on same samples using the specific primers (RVD VP6-DF, RVD VP6-DR) with the optimized reaction conditions (initial denaturation at 95°C for 5 min, cyclic denaturation at 94°C for 10 sec, annealing at 54°C for 20 sec and extension at 72°C for 30 sec for 35 cycles and a final extension temperature–time combination of 72°C for 7 min). PCR reaction was carried out containing 6 µl 2× PCR master mix, 1 µl forward primer, 1 µl reverse primer, 3 µl NFW and 1 µl of cDNA was added to make final volume to 12 µl. The PCR components were mixed and spun briefly.

Amplification of VP4 and VP7 genes of AvRVD: Conditions for amplification of two structural genes (full length VP7 and partial length VP4) of AvRVD were in-house optimized. Twenty seven samples were screened. PCR reaction was carried out containing 6 µl 2× PCR master mix, 1 µl forward primer, 1 µl reverse primer, 3 µl NFW and 1 µl cDNA was added to make final volume of 12 µl. The PCR components were mixed and spun shortly. Reaction conditions were similar to VP6 gene based PCR for AvRVD.

Amplification of NSP4 and NSP5 gene of AvRVD: Twenty seven samples were screened for NSP4 and NSP5

Table 1. Primers for the amplification of Group A and Group D avian rotavirus

Gene	Primers	Sequence (5' to 3')	Position	Amplicon size (bp)	References
VP6	ARVA6-1F	CACCACGACTTATGCAGAGA	709-728	493 bp	Otto *et al.* 2012
	ARVA6-1R	CTCCGAATGGATGCTACTGT	1201-1182		
	RVD VP6-DF	GCTATACATTTCGCTGCATTTG	580-601	185 bp	Malik *et al.* 2014
	RVD VP6-DR	TGGCCAATAGTGTGTGGCAGCT	765-744		
VP7	RVD-VP7/P1 FP	AGGCATCTTAACCATATAGGA	24-44	993 bp	Malik 2020
	RVD-VP7/P1 RP	ATATCATAACTATTGGCGAAC	1017-997		(Unpublished)
VP4	RVD-VP4/P1 FP	TTTTAAAGCACATGAGACTAG	4-24	1038 bp	
	RVD-VP4/P1 RP	TTACGACGATCTATTTCACTA	1042-1022		
NSP4	RVD-NSP4 FP	AATTTATTAGTTGAATCCATC	9-30	756 bp	
	RVD-NSP4 RP	GGTCACAATTTATTAGTTTCC	765-744		
NSP5	RVD-NSP5 FP	GGTTTTAAATTGCTACTAGAG	1-21	652 bp	
	RVD-NSP5 RP	GGTCACCCTAGAGTACATACA	653-633		

genes of AvRVD. PCR reaction was carried out containing 25 µl 2× PCR master mix, 2 µl forward primer, 2 µl reverse primer, 20 µl NFW and 1 µl of cDNA was added to make final volume to 50 µl. The PCR components were mixed and spun briefly. Reaction conditions were similar to VP6 gene based PCR for AvRVD.

The specific PCR amplicons were visualized in ethidium bromide stained 2% agarose gel and documented in Gel-Doc assembly (Uvitech).

Cloning: Six positive samples for NSP5 gene were taken for cloning. The specific RT-PCR amplicon (652 bp) was gel-purified by GeneJET Gel extraction kit (Thermo Scientific) and cloned into pJet1.2/blunt cloning vector (Thermo Scientific). The ligation reaction was set on ice consisting of 10 µl 2× reaction buffer (50 ng/µl), 1 µl purified PCR product (blunt ended), 1 µl pJet1.2 blunt cloning vector, 19 µl NFW, 1 µl T4 DNA ligase in a reaction volume of 20 µl as per the given protocol. *E. coli* top 10 competent cell were transformed and recombinants selected.

Nucleotide sequence analysis and phylogenetic study: Sequencing of the cloned product was done from Eurofins Pvt. Ltd., Gurugram. Sequence identity calculation was performed using the Clustal W program available in the Megalign software of the DNASTAR software package. Sequence identities of the NSP5 gene of RVD were calculated in between available RVD, RVF, RVG gene sequences. To elucidate the genetic identity of the RVD recognized in the current study, phylo-dendrogram was prepared in Molecular Evolutionary Genetic Analysis (MEGA) software (v 6.06).

RESULTS AND DISCUSSION

Out of 100 samples screened for AvRVA-VP6 gene, none of the sample was positive for AvRVA. Out of 20 fecal samples collected from the Mathura region, 5 (25%) samples were positive in diagnostic analysis of AvRVD-VP6 gene. Among the 80 intestinal samples, 22 (27.5%) were positive for AvRVD-VP6 gene. An amplicon of 185 bp was obtained for VP6 gene of AvRVD (Fig. 1). No sample was found to amplify VP4 and VP7 genes of AvRVD. Among 27 samples screened, six samples (F14, F16, F19, D1, D3 and D4) were positive for NSP5 gene giving an amplicon 652 bp size (Fig. 2).

Prevalence of AvRD: Prevalence of AvRVD was 27% in the current study. Twenty four samples were from organised farm and three from unorganised sector. Most (86%) of intestinal samples positive were diarrheic. The samples were watery in consistency, foul smelling and yellow coloured. Most samples were collected in the winter months (Nov-Dec). Rotaviruses have been mostly reported in winter months (Basera *et al.* 2010). Both diarrheic and non-diarrheic samples have been found to be positive for rotavirus (Savita *et al.* 2008).

In India, the presence of AvRVs belonging to Group A has been reported from limited geographical areas originating from northern and central parts of the country

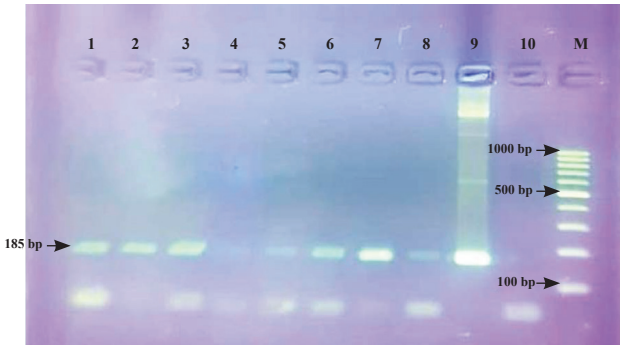

Fig. 1. Agarose gel electrophoresis of amplification products of VP6 gene of AvRVD. Lane 1, 2, 3, 4, 5, 6, 7 and 8: Positive samples; Lane 9: Positive control; Lane 10: Negative control; Marker: 100 bp DNA ladder.

(Wani *et al.* 2003, Minakshi *et al.* 2004, Savita *et al.* 2008). Wani *et al.* (2003) first time detected AvRVA in diarrheic chicken by sandwich ELISA and PAGE in India. The reports on RVD in India are limited. The first report on RVD came from central India in 2008, based on the specific electrophoretic migration pattern of RVD i.e., 5:2:2:2 (Savita *et al.* 2008). In 2010, RVD was detected in western parts (Maharashtra) of India, where 7.84% of poultry were positive and electropherotyping revealed 5:2:2:2 migration patterns (Niture *et al.* 2010). Using RT-PCR, in 2011–2012, a 6.04% positivity rate was confirmed for RVD in chickens from northern states of India (Kattoor *et al.* 2013a). Using the same RT-PCR, recent (2012–2017) screening data revealed that this percent positivity in northern India has increased nearly fourfold over the last five years (Deol *et al.* 2017). In central part of India, Group D RVs have been reported to be more prevalent (77.8%) than RVA (22.2%) along with presence of strains that are genetically diverse in nature (Savita *et al.* 2008).

In the south-eastern part of Asia, the prevalence of RVs in avian species varied from 2.9% to 22.5% (Ahmed and Ahmed 2006, Karim *et al.* 2007). A study in Brazil revealed 53.8% prevalence of rotavirus from broilers, layers and broiler breeders (Beserra and Gregory 2014).

Nucleotide sequence analysis: The six RVD isolates F14, F16, F19, D1, D3 and D4 showed 99.4 to 100% sequence similarity among themselves at the nucleotide level. The

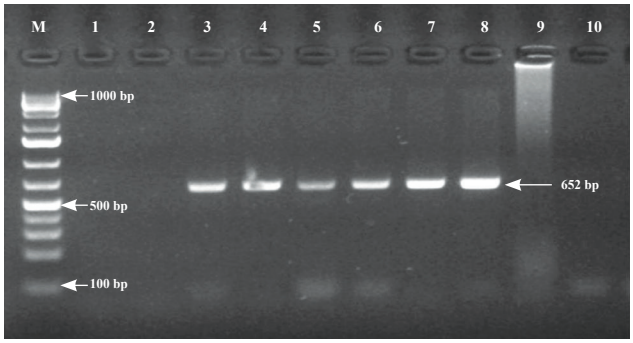

Fig. 2. Agarose gel electrophoresis of amplification products of NSP5 gene of AvRVD. Marker: 100 bp DNA ladder; Lane 3 to 8: positive samples; Lane 9: negative control.

per cent nucleotide similarity of the sequences with chicken RVD isolates was in the range 78.2 – 85.1%. All the isolate showed the highest similarity of 85% with the chicken isolate D62 (KM254201) from South Korea followed by Germany (81.5%) and Australia (78.2%) (Fig. 3).

Phylogenetic study: The sequencing of phylogenetic studies gives an idea about the origin, type of species involved and relationship of local isolates with the isolates of different part of world. Further, the phylogenetic analysis based on RVD NSP5 gene was performed for the six isolates. The phylogenetic analysis exhibited three different clades in the dendrogram. All the six isolates (F14, F16, F19, D1, D3 and D4) clustered together and form a separate clade with the isolates from Australia, Germany and South Korea. In the phylogenetic tree, the RVA formed a separate clade while RVF and RVG were clustered in a separate group (Fig. 4).

First sequence-confirmed RVD infection in 1–2 week old broiler chicks was reported from northern India and based on partial VP6 gene of type D rotavirus, Indian strains revealed higher homology with avian Group D rotaviruses from Brazil, Germany, Netherlands, Bangladesh and UK, both at the nucleotide (87.2–89.6%) and amino acid (98.7–99.5%) levels (Kattoor *et al.* 2013b). Pauly *et al.* (2017)

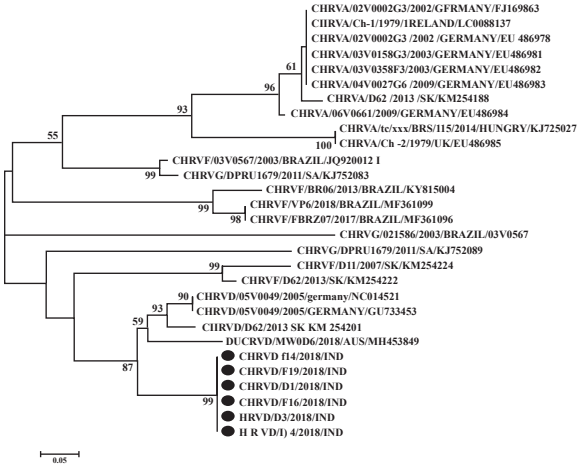

Fig. 4. Phylogenetic analysis of rotaviruses based on the RVD NSP5 gene. The present study RVD NSP5 sequences (n=6) are indicated by the dark black circle "●". Scale bar indicates nucleotide substitutions per site.

reported little diversity (>98% nucleotide identity for VP6 and >99% nucleotide identity for VP7) of RVD sequences that were clearly distinct from RVA. Trojnar *et al.* (2013) presented complete genomes of RVA strain 03V0002E10

Pair Distances of Untitled ClustalW (Slow/Accurate, IUB)

Percent Similarity in upper triangle

Percent Divergence in lower triangle

	CHRVD_f14_2018_IND	CHRVD_F19_2018_IND	CHRVD_D1_2018_IND	CHRVD_F16_2018_IND	CHRVD_D3_2018_IND	CHRVD_D4_2018_IND	CHRVD_SK_KM254201	CHRVF_VP6_2018_BRAZIL_MF361099	CHRVD_05V0049_2005_germany_NC014521	CHRVD_05V0049_2005_GERMANY_GU733453	DUCRVD_MW0D6_2018_AUS_MH453849	CHRVF_D11_2007_SK_KM254224	CHRVF_D62_2013_SK_KM254222	CHRVF_FBRZ07_2017_BRAZIL_MF361096	CHRVF_BR06_2013_BRAZIL_KY815004	CHRVF_03V0567_2003_BRAZIL_JQ920012	CHRVG_DPRU1679_2011_SA_KJ752089	CHRVG_DPRU1679_2011_SA_KJ752083	CHRVG_021586_2003_BRAZIL_03V0567	CHRVA_D62_2013_SK_KM254188	CHRVA_tc_xxx_BRS_115_2014_HUNGRY_KJ725027	CHRVA_06V0661_2009_GERMANY_EU486984	CHRVA_04V0027G6_2009_GERMANY_EU486983	CHRVA_03V0358F3_2003_GERMANY_EU486982	CHRVA_03V0158G3_2003_GERMANY_EU486981	CHRVA_02V0002G3_2002_GERMANY_EU486978	CHRVA_Ch-2_1979_UK_EU486985	CHRVA_02V0002G3_2002_GERMANY_FJ169863
CHRVD_f14_2018_IND	***	99.5	99.5	99.5	99.4	99.4	84.7	5.9	81.1	81.1	78.2	6.7	7.2	5.9	6.1	10.8	18.1	6.2	12	8.5	3.9	4	3.8	3.8	3.8	3.8	3.9	3.7
CHRVD_F19_2018_IND		***	99.5	99.4	99.7	99.8	85.1	5.8	81.2	81.2	78.2	6.7	7.2	5.8	6	10.6	18	6.2	11.8	8.5	3.8	4	3.8	3.8	3.8	3.8	3.8	3.7
CHRVD_D1_2018_IND			***	99.7	99.7	99.7	85.1	5.7	80.8	80.8	78.2	6.7	7.2	5.7	5.9	10.4	17.7	6.2	11.6	8.5	3.8	4	3.8	3.8	3.8	3.8	3.8	3.6
CHRVD_F16_2018_IND				***	99.8	99.8	85.1	5.7	80.9	80.9	78.2	6.7	7.2	5.7	5.9	10.4	17.7	6.2	11.6	8.5	3.8	4	3.8	3.8	3.8	3.8	3.8	3.6
CHRVD_D3_2018_IND					***	100	85.1	5.9	81.5	81.5	78.2	6.7	7.2	5.9	6.1	10.7	18.2	6.2	12	8.5	3.9	4	3.8	3.8	3.8	3.8	3.9	3.7
CHRVD_D4_2018_IND						***	85.1	5.9	81.5	81.5	78.2	6.7	7.2	5.9	6.1	10.7	18.2	6.2	12	8.5	3.9	4	3.8	3.8	3.8	3.8	3.9	3.7
CHRVD_D62_2013_SK_KM254201							***	5.3	79.8	79.8	57.2	5.1	8.7	5.1	5.1	11.8	9.7	5.8	10.4	14.7	9.3	9.7	6.4	6.4	6.4	6.4	9.3	6.4
CHRVF_VP6_2018_BRAZIL_MF361099								***	4	4	14.4	5.5	6.9	97.2	94.7	6.2	2.4	6.7	5.4	9.2	3.6	4	3.8	3.8	3.8	3.8	4	3.9
CHRVD_05V0049_2005_germany_NC014521									***	100	82.7	4.2	7.6	4.2	4	9.4	7.4	8.1	3.4	8.5	21.7	3.8	3.7	3.7	3.7	3.7	3.8	6.7
CHRVD_05V0049_2005_GERMANY_GU733453										***	82.7	4.2	7.6	4.2	4	9.4	7.5	8.1	3.4	8.5	21.2	3.8	3.7	3.7	3.7	3.7	3.8	3.4
DUCRVD_MW0D6_2018_AUS_MH453849											***	13.2	6.6	11.1	7	21.8	7.8	17.7	9.9	19.8	9.9	9.9	9.5	9.5	9.5	9.5	9.9	9.5
CHRVF_D11_2007_SK_KM254224												***	76.4	6.9	5.5	5.5	6.9	3	7.6	4.8	6	9.5	9.2	9.2	9.2	9.2	6	9.2
CHRVF_D62_2013_SK_KM254222													***	16.4	7.1	13.9	2.6	6.7	7.9	15.8	4.6	6.9	8.3	13.9	6.7	6.7	4.6	6.7
CHRVF_FBRZ07_2017_BRAZIL_MF361096														***	93.5	5.5	3.8	5.9	3.5	9.6	3.6	4	3.8	3.8	3.8	3.8	4	3.9
CHRVF_BR06_2013_BRAZIL_KY815004															***	6.8	2.2	7.1	6.9	14	3.4	3.8	6.5	6.5	6.5	6.5	3.8	3.7
CHRVF_03V0567_2003_BRAZIL_JQ920012																***	7.1	94.1	4.3	18.8	3.2	12.9	4	4	4	4	3.3	3.7
CHRVG_DPRU1679_2011_SA_KJ752089																	***	7.6	4.2	8.1	6.3	2.9	6.7	2.6	9.6	9.6	7	8.6
CHRVG_DPRU1679_2011_SA_KJ752083																		***	4.3	18.8	8.7	9.7	7.7	7.7	7.7	7.7	8.7	7.6
CHRVG_021586_2003_BRAZIL_03V0567																			***	10.3	4.8	4.3	4.3	4.3	4.3	4.3	5.3	3.9
CHRVA_D62_2013_SK_KM254188																				***	65.1	92.6	94.1	94.1	94.1	94.1	65.1	94.1
CHRVA_tc_xxx_BRS_115_2014_HUNGRY_KJ725027																					***	73.8	72.2	72.6	72.4	72.4	99.8	75.4
CHRVA_06V0661_2009_GERMANY_EU486984																						***	94.9	94.9	95.1	95.1	72.9	95.1
CHRVA_04V0027G6_2009_GERMANY_EU486983																							***	99.7	99.8	99.8	72.4	99.8
CHRVA_03V0358F3_2003_GERMANY_EU486982																								***	99.8	99.8	72.7	99.8
CHRVA_03V0158G3_2003_GERMANY_EU486981																									***	100	72.6	100
CHRVA_02V0002G3_2002_GERMANY_EU486978																										***	72.6	100
CHRVA_Ch-2_1979_UK_EU486985																											***	68.9
CHRVA_02V0002G3_2002_GERMANY_FJ169863																												***
CHRVA_Ch-1_1979_IRELAND_LC088137																												

Fig. 3. Nucleotide sequence analysis.

from turkey (*Meleagris gallopavo*) and RVA strain 10V0112H5 from pheasant (*Phasianus colchicus*). Most genome segments of both strains were related closely to those of avian RVAs. The present study involved detection of AvRVD by NSP5 gene sequence based analysis, whereas, it has not been targeted earlier. The presence of AvRVD in present study in organized farm in comparison to unorganized sector indicates the susceptibility of farms for built up infections and use of NSP5 gene for sequence-based identification.

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