Concurrent infection of Bluetongue and Peste-des-petits-ruminants virus in small ruminants in Haryana state of India

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Concurrent infection of Bluetongue and Peste-des-petits-ruminants virus in small ruminants in Haryana state of India

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Running title: Co-infection of BTV and PPRV in Haryana, India

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Bluetongue, Peste-des-petits-ruminants virus, Haryana, India, Epidemiology.

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Summary

Bluetongue (BT) and Peste-des-petits-ruminants (PPR), are major transboundary diseases of small ruminant, which are endemic in India. Testing of bluetongue virus (BTV) and peste-des-petits-ruminants virus (PPRV), from recent outbreaks (2015–2016) in different regions of Haryana state of India revealed that 27.5 % of the samples showed the presence of dual infection of BTV and PPRV. Analysis of Seg-2 of BTV (the serotype determining protein) showed the presence of BTV-12w in several isolates. However, analysis of N gene fragment amplicons showed that viruses belong to lineage IV most closely related to a pathogenic strain of PPRV from Delhi. This is the first report of co-circulation of PPRV lineage IV and bluetongue virus serotype 12 in the state.

Contents

Haryana is one of the 29 states in India, situated in North and its Livestock population consist of 6.08 million buffalos, 1.80 million cattle, 0.36 million sheep, and 0.36 million goats which represents the main livelihood for the majority of the rural population of Haryana...

Bluetongue (BT) is an arthropod-transmitted, WOAH notifiable, viral disease of ruminants and certain other animals. Bluetongue virus (BTV) is the 'type species' of the genus *Orbivirus* within the *Reoviridae* family (Mertens et al., 2005). Twenty-seven serotypes have been recognized for this virus so far (Hofmann et al., 2008, Maan et al., 2011, Zientara et al., 2014). There is evidence of two additional putative serotypes (Maan et al., 2016).The BTV genome consists of ten segments of double-stranded RNA surrounded by three concentric protein layers without any lipid envelope (Mertens et al., 2009).

In India several BTV serotypes −1e, −2e, −2w −3e, −5w, −9e, −10w, −12w, −16e, −21e −23e and −24w are currently circulating (Maan et al., 2015b, Rao et al., 2014). Only two BTV serotypes (BTV-1 and BTV-4) have been previously isolated from Haryana state in 1985 (Jain et al., 1986) and in 2001 (Uppal and and Vasudevan, 1980). However, there is serological evidence of BTV-2, -8, -12 and -16 from Haryana (Prasad et al., 2009).

Similarly Peste-des-petits-ruminants is another serious, highly contagious, WOAH notifiable and economically important transboundary infectious disease of sheep and goats, which is associated with high mortality and morbidity. Although, a live attenuated PPR vaccine based on the PPRV/Sungri/96 strain is being used in India even then, PPR outbreaks are being

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reported in small ruminants recurrently throughout the year (Balamurugan et al., 2012, Kumar et al., 2014).

This disease is caused by peste-des-petits-ruminants virus (PPRV), which belongs to the genus *Morbillivirus* of the family *Paramyxoviridae* (sub family *Paramyxovirinae*) under the order *Mononegavirales* (Lefevre and Diallo, 1990). The PPRV genome encodes six structural (N, P, M, F, H and L) and two nonstructural (C and V) proteins (Mahapatra et al., 2006). The PPRV is genetically grouped into four lineages (I, II, III, and IV) based on the partial F and N gene sequences analyses. Lineages I–III have been found to circulate in Africa, while lineage IV is generally reported from Asia (Banyard et al., 2010, Kwiatek et al., 2011). However, over the last decade, there have been reports of the presence of the Asian lineage of PPRV in several African countries (Kwiatek et al., 2011, De Nardi et al., 2012).

Spread of both PPR and BT diseases to a number of new geographical areas with involvement of various lineage of PPRV and multiple topotypes and serotypes of BTV is a cause of global concern, thus prompting us to reassess the epidemiological situation of BTV and PPRV in Haryana.

During this study, a total of 348 clinical samples (240 from sheep and 108 from goats) consisting of various tissues (spleen, trachea, lung, liver and lymph nodes), blood, nasal, ocular and rectal swabs were collected from different villages of Haryana between 2015 and 2016 from the outbreaks of PPR and/or BT that occurred throughout the year irrespective of the season (data from March 2015 to July 2016). This could be because the winters were very mild and also summers were not that harsh during the period under study. Both sheep and goat showed similar disease pattern and the clinical signs in the affected animals included high rise of temperature (105^0F-106^0F), depression, anorexia, hyperemia of mucus membranes of lips and nostrils, purulent nasal discharge, dermatitis, wool break, diarrhea and lameness. Most of the affected animals were between 3 and 12 months of age. Goats and sheep were kept together in these areas where they shared grazing land and drinking water sources. The samples were collected from migratory flocks and from the flocks kept by local farmers involving native breeds of sheep (Nali and Munjal) and goats (Beetal). The location of each sample was recorded using the standard proforma devised for the Orbivirus reference collection (ORC) at the Pirbright Laboratory, UK (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-1.htm).

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Total RNA was extracted from the samples (nasal/ocular/rectal cotton swabs/tissues/ cell culture supernatants) either using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions or using Trizol Reagent (Life Technologies Inc) (Attoui et al., 2000). Additionally, RNA from uninfected tissue culture supernatants and uninfected sheep and goat blood was also extracted for use as negative controls.

These samples were analysed for the presence of BTV RNA and PPRV RNA using Seg-9 (Maan et al., 2015a) and Seg-10 (Orru et al., 2006) specific and N gene specific qRT-PCR assays respectively (Batten et al., 2011). 253 samples (72.70 %) were positive for the presence of BTV and 137 samples (39.36 %) for PPRV. In 96 (27.5 %) of cases there were dual infection of BTV and PPRV.

The samples with Ct values less than 30 were taken further for virus isolations in respective cell lines. Majority of the samples that were positive for BTV were converted into isolates in KC and BHK cells. BTV isolates (e.g. IND2015/340 originating from field sample IND2015/338) produced characteristic CPE in BHK-21 cells (granulation, rounding, detachment and degeneration of cells). Some of the selective samples showing concurrent infection of BTV and PPRV were passaged directly in Vero SLAM cells, as previously described (Chinnakannan et al., 2013) for isolation of PPRV. PPRV isolates generated (e.g. PPRV/IND2015/02 originating from same field sample IND2015/338) also produced characteristic CPE in Vero SLAM cells, which was characterised by rounding and ballooning of cells followed by aggregation of cells and formation of fusion mass and syncytia. Cell lysis was also observed in some cases.

The BTV isolates were serotyped using qRT-PCR assays either using a panel of type specific qRT-PCR assays targeting Seg-2 (Maan et al., 2016), which revealed the presence of BTV-12 in all of isolates from different regions of Haryana showing concurrent infection of BTV and PPRV. Conventional gel based Seg-2 specific type assays confirmed the presence of BTV-12w in the isolates that were tested (Maan et al., 2012).

Seg-2 of BTV-12 isolates was amplified in four overlapping fragments using the primers listed in table 1. Sequencing of these Seg-2 amplicons on ABI capillary sequencer 3130 using a 'Big dye cycle sequencing kit' followed by assembly using Lasergene software ver. 5.0, has confirmed the presence of serotypes 12 in these isolates. Comparison of full length BTV-12 Seg-2 nucleotide sequences (from isolate IND2015/340 – accession no KX905151) and partial sequences from the other BTV-12 isolates (not listed here) in MEGA software version 6 (Tamura

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124 et al., 2013), showed that these were very closely related to each other (99.9% nt identity) and to
125 another Indian isolate from southern India BTV-12/INDAPADBNMO1/11 (Accession no.
126 KC662613) (99.3%)(Rao et al., 2013) (Figure 1). Seg-2 of IND2015/340 showed 97.1% nt
127 sequence identity with the reference strain of BTV-12 from South Africa (Accession no.
128 AJ585133) and Kenya (Accession no. AJ585185), and share 95.8% to 96.6% nt sequence
129 identity to strains from Taiwan (Accession nos. GU390659 and AB686216). On the contrary,
130 Seg-2 of IND2015/340 showed greater variation (87.6% nt identity) from BTV-12 strain from
131 France Guyane (Accession no. KC633278).

132 Sequencing of partial N gene amplicons (generated using the primers listed in table 1)
133 from PPRV samples and isolates has confirmed the results of real time RT-PCR assays for the
134 presence of PPRV RNA. Partial N gene based phylogeny (1576 bp of PPRV/IND2015/02
135 [accession no - KX905152]), revealed that most of the PPRV strains collected from Haryana
136 between 2015 and 2016 (n = 137) grouped together in lineage IV very closely related with other
137 strains from India (IND/Delhi/2016/05 – Accession no. KX033350, IND/TN/GIN/2014/01 –
138 Accession no. KT270355 and India/TN/Gingee/2014 – Accession no. KR261605) with 98.4% to
139 98.9% nt identity in N gene supported by a bootstrap value of >90% (Figure 2). However, the
140 % nt identity in N gene with the vaccine strain being used in India (Sungri 1996 MSD -
141 Accession no. - KJ867542) is 96.8%. The phylogeny inferred for BTV Seg-2 and PPRV N gene
142 with the distance methods were consistent with those of the character-based analysis.

143 The findings of this study relating to BT and PPR surveillance in Haryana over a more
144 than one year period (January 2015 – July 2016) have confirmed the widespread distribution of
145 BT and PPRV throughout the province. The concurrent infection of BTV and PPRV has been
146 reported in Haryana. This is the first report of circulation of PPRV lineage IV and bluetongue
147 virus serotype 12 in the state.

148

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Conflict of interest

The authors declare no conflict of interest.

Tables:

Table 1. Details of primers used for amplification of N gene of Peste-des-petits-ruminants virus (PPRV) and VP2 gene of Bluetongue virus (BTV) for use in RT-PCR assays.

Figure legends

Figure 1: Neighbour-joining tree showing the relationships between Seg-2 of IND2015/340 with the twenty nine reference strains of different BTV serotypes. The tree was constructed using distance matrices, generated using the p-distance determination algorithm in MEGA 6.0 (500 bootstrap replicates). The bootstrap values are indicated at the evolutionary branching points. The tree based on the character based method (Maximum likelihood) showed very similar topology.

Figure 2: Neighbour-joining tree showing the relationships between N gene sequence of PPRV/IND2015/338 with the other global strains of Morbiliviruses. The tree was constructed with partial (1576 bp) N gene sequences, using the p-distance determination algorithm in MEGA 6.0 (500 bootstrap replicates). The bootstrap values are indicated at the evolutionary branching points. The tree based on the character based method (Maximum likelihood) showed very similar topology.

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Haryana is one of the 29 states in India, situated in North and its Livestock population consist of 6.08 million buffalos, 1.80 million cattle, 0.36 million sheep, and 0.36 million goats which represents the main livelihood for the majority of the rural population of Haryana...

Bluetongue (BT) is an arthropod-transmitted, WOAH notifiable, viral disease of ruminants and certain other animals. Bluetongue virus (BTV) is the 'type species' of the genus *Orbivirus* within the *Reoviridae* family (Mertens et al., 2005). Twenty-seven serotypes have been recognized for this virus so far (Hofmann et al., 2008, Maan et al., 2011, Zientara et al., 2014). There is evidence of two additional putative serotypes (Maan et al., 2016).The BTV genome consists of ten segments of double-stranded RNA surrounded by three concentric protein layers without any lipid envelope (Mertens et al., 2009).

In India several BTV serotypes −1e, −2e, −2w −3e, −5w, −9e, −10w, −12w, −16e, −21e −23e and −24w are currently circulating (Maan et al., 2015b, Rao et al., 2014). Only two BTV serotypes (BTV-1 and BTV-4) have been previously isolated from Haryana state in 1985 (Jain et al., 1986) and in 2001 (Uppal and and Vasudevan, 1980). However, there is serological evidence of BTV-2, -8, -12 and -16 from Haryana (Prasad et al., 2009).

Similarly Peste-des-petits-ruminants is another serious, highly contagious, WOAH notifiable and economically important transboundary infectious disease of sheep and goats, which is associated with high mortality and morbidity. Although, a live attenuated PPR vaccine based on the PPRV/Sungri/96 strain is being used in India even then, PPR outbreaks are being

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reported in small ruminants recurrently throughout the year (Balamurugan et al., 2012, Kumar et al., 2014).

This disease is caused by peste-des-petits-ruminants virus (PPRV), which belongs to the genus *Morbillivirus* of the family *Paramyxoviridae* (sub family *Paramyxovirinae*) under the order *Mononegavirales* (Lefevre and Diallo, 1990). The PPRV genome encodes six structural (N, P, M, F, H and L) and two nonstructural (C and V) proteins (Mahapatra et al., 2006). The PPRV is genetically grouped into four lineages (I, II, III, and IV) based on the partial F and N gene sequences analyses. Lineages I–III have been found to circulate in Africa, while lineage IV is generally reported from Asia (Banyard et al., 2010, Kwiatek et al., 2011). However, over the last decade, there have been reports of the presence of the Asian lineage of PPRV in several African countries (Kwiatek et al., 2011, De Nardi et al., 2012).

Spread of both PPR and BT diseases to a number of new geographical areas with involvement of various lineage of PPRV and multiple topotypes and serotypes of BTV is a cause of global concern, thus prompting us to reassess the epidemiological situation of BTV and PPRV in Haryana.

During this study, a total of 348 clinical samples (240 from sheep and 108 from goats) consisting of various tissues (spleen, trachea, lung, liver and lymph nodes), blood, nasal, ocular and rectal swabs were collected from different villages of Haryana between 2015 and 2016 from the outbreaks of PPR and/or BT that occurred throughout the year irrespective of the season (data from March 2015 to July 2016). This could be because the winters were very mild and also summers were not that harsh during the period under study. Both sheep and goat showed similar disease pattern and ~~T~~the clinical signs in the affected animals included high rise of temperature (105^0F-106^0F), depression, anorexia, hyperemia of mucus membranes of lips and nostrils, purulent nasal discharge, dermatitis, wool break, diarrhea and lameness. Most of the affected animals were between 3 and 12 months of age. Goats and sheep were kept together in these areas where they shared grazing land ~~grazed together~~ and ~~shared the same~~ drinking water sources. The samples were collected from migratory flocks and from the flocks kept by local farmers involving native breeds of sheep (Nali and Munjal) and goats (Beetal). The location of each sample was recorded using the standard proforma devised for the Orbivirus reference collection (ORC) at the Pirbright Laboratory, UK (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-1.htm).

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9 94 Total RNA was extracted from the samples (nasal/ocular/rectal cotton swabs/tissues/ cell culture supernatants) either using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions or using Trizol Reagent (Life Technologies Inc) (Attoui et al., 2000). Additionally, RNA from uninfected tissue culture supernatants and uninfected sheep and goat blood was also extracted for use as negative controls.

These samples were analysed for the presence of BTV RNA and PPRV RNA using Seg-9 (Maan et al., 2015a) and Seg-10 (Orru et al., 2006) specific and N gene specific qRT-PCR assays respectively (Batten et al., 2011). 253 samples (72.70 %) were positive for the presence of BTV and 137 samples (39.36 %) for PPRV. In 96 (27.5 %) of cases there were dual infection of BTV and PPRV.

The samples with Ct values less than 30 were taken further for virus isolations in respective cell lines. Majority of the samples that were positive for BTV were converted into isolates in KC and BHK cells. BTV isolates (e.g. IND2015/340 originating from field sample IND2015/338) produced characteristic CPE in BHK-21 cells (granulation, rounding, detachment and degeneration of cells). Some of the selective samples showing concurrent infection of BTV and PPRV were passaged directly in Vero SLAM cells, as previously described (Chinnakannan et al., 2013) for isolation of PPRV. PPRV isolates generated (e.g. PPRV/IND2015/02 originating from same field sample IND2015/338) also produced characteristic CPE in Vero SLAM cells, which was characterised by rounding and ballooning of cells followed by aggregation of cells and formation of fusion mass and syncytia. Cell lysis was also observed in some cases.

The BTV isolates were serotyped using qRT-PCR assays either using ~~BTV typing kits available from LSI (now Life Technologies Inc) or~~ a panel of type specific qRT-PCR assays targeting Seg-2 (Maan et al., 2016), which revealed the presence of BTV-12 in all of isolates from different regions of Haryana showing concurrent infection of BTV and PPRV. Conventional gel based Seg-2 specific type assays confirmed the presence of BTV-12w in the isolates that were tested (Maan et al., 2012).

Seg-2 of BTV-12 isolates was amplified in four overlapping fragments using the primers listed in table 1. Sequencing of these Seg-2 amplicons on ABI capillary sequencer 3130 using a 'Big dye cycle sequencing kit' followed by assembly using Lasergene software ver. 5.0, has confirmed the presence of serotypes 12 in these isolates. Comparison of full length BTV-12 Seg-2 nucleotide sequences (from isolate IND2015/340 – accession no KX905151) and partial

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sequences from the other BTV-12 isolates (not listed here) in MEGA software version 6 (Tamura et al., 2013), showed that these were very closely related to each other (99.9% nt identity) and to another Indian isolate from southern India BTV-12/INDAPADBNMO1/11 (Accession no. KC662613) (99.3%)(Rao et al., 2013) (Figure 1). Seg-2 of IND2015/340 showed 97.1% nt sequence identity with the reference strain of BTV-12 from South Africa (Accession no. AJ585133) and Kenya (Accession no. AJ585185), and share 95.8% to 96.6% nt sequence identity to strains from Taiwan (Accession nos. GU390659 and AB686216). On the contrary, Seg-2 of IND2015/340 showed greater variation (87.6% nt identity) from BTV-12 strain from France Guyane (Accession no. KC633278).

Sequencing of partial N gene amplicons (generated using the primers listed in table 1) from PPRV samples and isolates has confirmed the results of real time RT-PCR assays for the presence of PPRV RNA. Partial N gene based phylogeny (1576 bp of PPRV/IND2015/02 [accession no - KX905152]), revealed that most of the PPRV strains collected from Haryana between 2015 and 2016 (n = 137) grouped together in lineage IV very closely related with other strains from India (IND/Delhi/2016/05 – Accession no. KX033350, IND/TN/GIN/2014/01 – Accession no. KT270355 and India/TN/Gingee/2014 – Accession no. KR261605) with 98.4% to 98.9% nt identity in N gene supported by a bootstrap value of >90%~~.~~ (Figure 2). However, the % nt identity in N gene with the vaccine strain being used in India (Sungri 1996 MSD - Accession no. - KJ867542) is 96.8%. The phylogeny inferred for BTV Seg-2 and PPRV N gene with the distance methods were consistent with those of the character-based analysis.

The findings of this study relating to BT and PPR surveillance in Haryana over a more than one year period (January 2015 – July 2016) have confirmed the widespread distribution of BT and PPRV throughout the province. ~~The concurrent infection of BTV and PPRV has been reported for the first time in Haryana.~~ The concurrent infection of BTV and PPRV has been reported in Haryana. This is the first report of circulation of PPRV lineage IV and bluetongue virus serotype 12 in the state.

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CARe scientist. The authors would also like to thank all collaborators of BBSRC-DBT project and various staff of Animal Husbandry Department of Haryana for their direct or indirect help.

Conflict of interest

The authors declare no conflict of interest.

Tables:

Table 1. Details of primers used for amplification of N gene of Peste-des-petits-ruminants virus (PPRV) and VP2 gene of Bluetongue virus (BTV) for use in RT-PCR assays.

Figure legends

Figure 1: Neighbour-joining tree showing the relationships between Seg-2 of IND2015/340 with the twenty nine reference strains of different BTV serotypes. The tree was constructed using distance matrices, generated using the p-distance determination algorithm in MEGA 6.0 (500 bootstrap replicates). The bootstrap values are indicated at the evolutionary branching points. The tree based on the character based method (Maximum likelihood) showed very similar topology.

Figure 2: Neighbour-joining tree showing the relationships between N gene sequence of PPRV/IND2015/338 with the other global strains of Morbiliviruses. The tree was constructed with partial (1576 bp) N gene sequences, using the p-distance determination algorithm in MEGA 6.0 (500 bootstrap replicates). The bootstrap values are indicated at the evolutionary branching points. The tree based on the character based method (Maximum likelihood) showed very similar topology.

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Table 1. Details of primers used for amplification of N gene of Peste-des-petits-ruminants virus (PPRV) and VP2 gene of Bluetongue virus (BTV) for use in RT-PCR assays.

Oligo Name **eastern (e) or western (w)**	**Oligo Sequence (5'-3')**	**Product name and Size (bp)**
PPRV N gene specific primers for use in conventional RT-PCR		
PPR/N/51-73F PPR/N/1627-1606R	CCATGGCTACTCTCCTTAAAAGC GCCGAGGAGATCCTTGTCGTTG	1576
BTV-12w VP2 gene specific primers for use in conventional RT-PCR		
BTV-12w/2/1-20F BTV-12w/2/1133-1113R	GTTAAAAGTTGCGAGGATGG GATYGTTCCTCGTAAGCAGG	A: 1133
BTV-12w/2/896-915F BTV-12w/2/1576-1555R	CAGATGTGGAATAGTGARGA ATCRTAATAATACGGCATAACT	B: 680
BTV-12w/2/1404-1442F BTV-12w/2/2250-2231R	ATGAYACGAAGTATAGAGA TCTCTRTGTAAATAGCTGAT	C: 846
BTV-12w/2/2105-2123F BTV-12w/2/2904R	GGRCGRTGATGGCGAG GTAAGTTGAAGCYGCGAGG	D: 799

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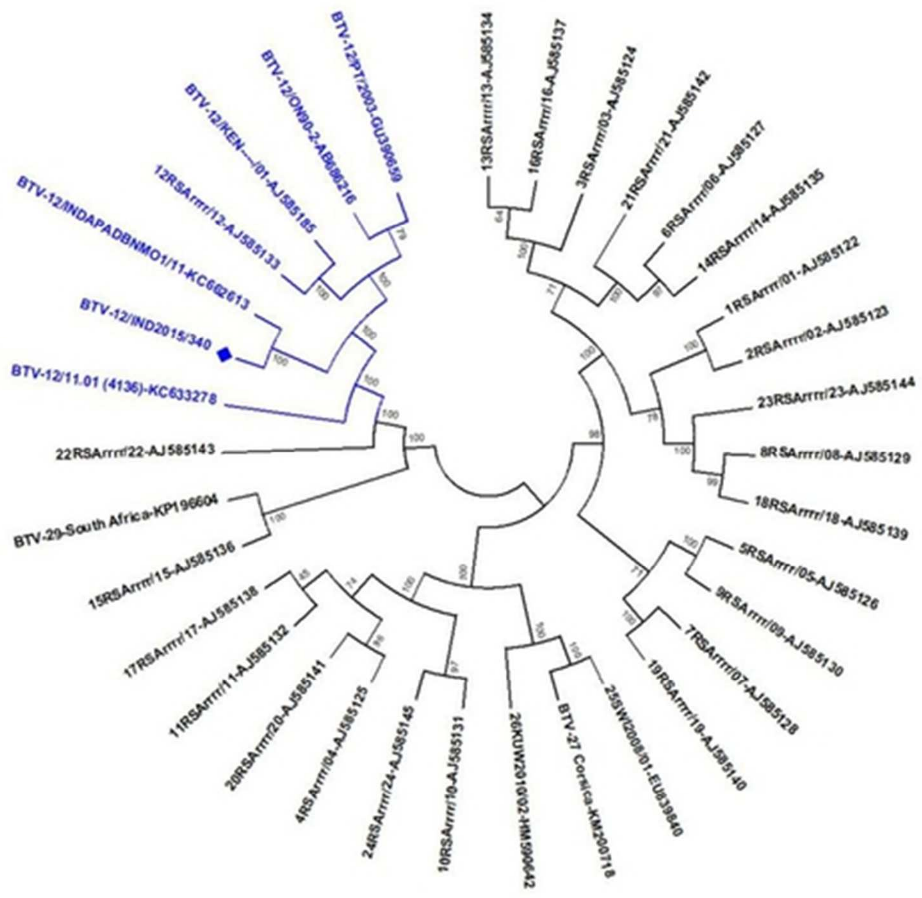

Figure 1: Neighbour-joining tree showing the relationships between Seg-2 of IND2015/340 with the twenty nine reference strains of different BTV serotypes. The tree was constructed using distance matrices, generated using the p-distance determination algorithm in MEGA 6.0 (500 bootstrap replicates). The bootstrap values are indicated at the evolutionary branching points. The tree based on the character based method (Maximum likelihood) showed very similar topology.

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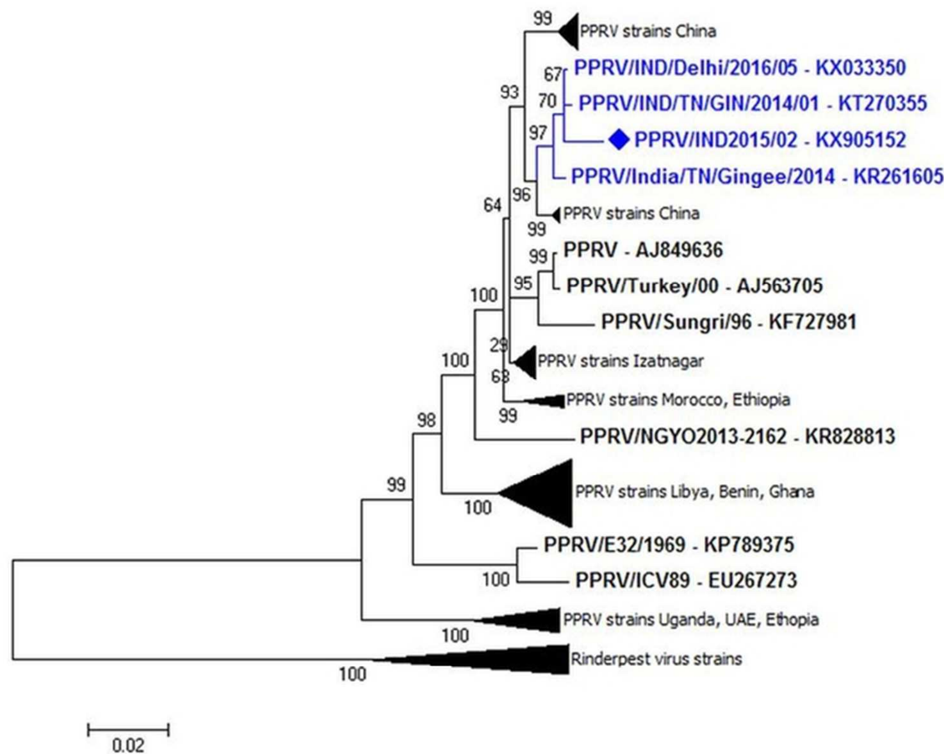

Figure 2: Neighbour-joining tree showing the relationships between N gene sequence of PPRV/IND2015/338 with the other global strains of Morbiliviruses. The tree was constructed with partial (1576 bp) N gene sequences, using the p-distance determination algorithm in MEGA 6.0 (500 bootstrap replicates). The bootstrap values are indicated at the evolutionary branching points. The tree based on the character based method (Maximum likelihood) showed very similar topology.

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