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Electrophoretic RNA genomic profiles of Brazilian *Picobirnavirus* (PBV) strains and molecular characterization of a PBV isolated from diarrheic calf

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ABSTRACT

Picobirnavirus (PBV) belongs to the family *Picobirnaviridae*. PBV are a group of emerging non-enveloped viruses, with a bisegmented double-stranded RNA genome that can infect a wide range of hosts. This study reports the occurrence of PBV in fecal samples from five Brazilian dairy cattle herds. From the 289 stool samples of individual calves analyzed by silver-stained polyacrylamide gel electrophoresis (ss-PAGE) the PBV was detected in 8.3 % (24/289), of which 10.2% (18/176) had diarrheic consistency. Of the 24 positive samples in ss-PAGE, 5 (20.8%) of them showed a small electrophoretic profile and 19 (79.2%) samples had large profile. From the 24 positives samples by ss-PAGE, 15 (62.5%) were successfully amplified (201 bp) using GI specific primers targeting the RdRp gene of PBV. The analysis of nucleotide identity matrix revealed that the bovine PBV strain identified in this study, showed the highest nucleotide identity (81%) with PBV strain detected in turkey (MD-2010/HM803965). This is the first nucleotide sequence of a bovine PBV strain in the American continent and the first detection of small genome profile of PBV-like strains in bovine hosts.

1. Introduction

Picobirnavirus (PBV), the only genus in the *Picornaviridae* family, has two species classified by the International Committee of Taxonomy of Viruses (ICTV): *Human picobirnavirus and Rabbit picobirnavirus*. The nomenclature of *Picobirnavirus* is based on the structural characteristics of the virus: the prefix "Pico" refers to the small size of the virion (35–40 nm in diameter) and "birna" due to the bi-segmented double-stranded RNA (dsRNA) of viral genome (King et al., 2011). The large RNA segment (or segment 1) encodes the capsid protein while the small segment (or segment 2) encodes the viral RdRp.

The estimated size of genomic segments of PBV is based on the typical specie A rotavirus electrophoretic migration profile in silver stained-polyacrylamide gel electrophoresis and (ss-PAGE). According to the migration profile of the two dsRNA segments in ss-PAGE, the PBV can be classified into two patterns: small or large genome profile. In large profile, the estimated sizes of the larger and smaller segments of PBV correspond to 2.7 kb and 1.9 kb, respectively. In small genome profile of PBV, the segment 1 has 2.2 kbp and the segment 2 has 1.2 kbp (Malik et al., 2014).

PBV has been classified into two genogroups: I (GI) and II (GII) due the genetic variability of segment 2. Most strains described belong to the GI PBV and infect a wide range of host species (Bhattacharya et al., 2007).

Several studies of detection of PBV have been described in fecal samples of several mammalian and reptilian species including humans, rats, calves, buffalo calves, pigs, horses, rabbits, giant anteaters, dogs, monkeys, orangutans and armadillo (Gallimore et al., 1995; Ludert et al., 1995; Haga et al., 1999; Buzinaro et al., 2003; Masachessi et al., 2007; Carruyo et al., 2008; Fregolente et al., 2009; Ganesh et al., 2011; Malik et al., 2011; Wang et al., 2012; Malik et al., 2014). In avian species, PBVs were detected in chickens (Alfieri et al., 1989; Tamehiro et al., 2003; Ribeiro Silva et al., 2014), turkey (Day et al., 2010), geese, emu, pelican and pheasant (Masachessi et al., 2007). However, regarding to molecular charac-

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terization of PBV from bovine host, only sequences from India are available in public DNA databases (Ghosh et al., 2009; Malik et al., 2011).

Although PBV was detected in feces of different hosts, its role as a causative agent of diarrhea is still controversial (Malik et al., 2014).

Fregolente and Gatti (2009) proposed a standardization of the PBV nomenclature. According to their recommendations, the PBV strain name starts with the genogroup (GI or GII), followed by PBV, host specie, three letter country code, strain name and year of isolation, separated by slashes.

The aim of this study was to report the frequency of PBV infection in dairy cattle herds and to perform the molecular characterization of a bovine PBV strain detected in fecal sample from a naturally infected calf. This is the first molecular detection by RT-PCR and phylogenetic analysis of bovine PBV in the American continent.

2. Methods

2.1. Fecal samples

From May 2012 to January 2014, were surveyed 289 calves between 5 and 60 days of age, from five dairy cattle herds located in five municipalities (Mariluz, Cafelândia, Toledo, Palotina and Marechal Cândido Rondon) of Paraná State, South of Brazil. Fecal samples were collected from the rectum of each calf and classified as diarrheic ($n = 176$) if they had liquid consistency and non-diarrheic ($n = 113$) if they had pasty or solid consistency. The distribution of diarrheic calves by total number of calves surveyed in each municipality was: Cafelândia (48/99); Mariluz (44/70); Palotina (31/44); Toledo (28/39); Marechal Cândido Rondon (25/37).

2.2. Fecal suspensions

Fecal suspensions were prepared at 10–20% in 0.01 M phosphate-buffered saline (PBS) pH 7.2, and centrifuged at 3,000 g for 15 min at 4 °C. The supernatants were used for RNA extraction. The samples were prepared either as 10% (w/v) suspensions of solid or pasty feces in Tris-calcium buffer (0.01 M Tris–HCl; 1.5 mM $CaCl_2$; pH 7.3) or as 50% (v/v) suspensions of liquid feces in Tris-calcium buffer and centrifuged at 3000 × *g* for 15 min at 4 °C. The supernatants were used for RNA extraction.

2.3. RNA extraction

Aliquots of 400 μL from fecal suspensions were treated with SDS at a final concentration of 1% (v/v), homogenized by vortexing and kept at 56 °C for 30 min. For RNA extraction a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinium isothiocyanate methods was performed according to Alfieri et al. (2006) with minor modifications. Briefly, 400 μL of phenol/chloroform/isoamyl alcohol (25:24:1) were added, vortexed and heated at 56 °C for 15 min. The mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was transferred into a new tube and processed by the silica/guanidinium isothiocyanate method (Boom et al., 1990). The RNA was eluted from the silica pellet with 50 μL of diethyl pyrocarbonate (DPEC) treated water (Invitrogen) by 15 min incubation at 56 °C and centrifugation at 10,000 × *g* for 10 min. The supernatant fraction was kept at −20 °C until use. Aliquots of DPEC water were included as negative control in all the RNA extraction procedures and specie A bovine rotavirus-positive fecal samples were included as positive control.

2.4. Silver stained-Polyacrylamide gel electrophoresis (ss-PAGE)

The dsRNA of PBV were analyzed by ss-PAGE at 7.5% according to the techniques described by Herring et al. (1982) and Pereira et al. (1983).

2.5. RNA denaturation and RT-PCR

All ss-PAGE PBV positive samples were submitted on RT-PCR. The assay was carried out using the primers PicoB25 (5′TGGTGTGGATGTTTC 3′) and PicoB43 (5′A(GA)TG(CT)TGGTCGAACT T 3′) that amplify a 201 bp fragment of the RdRp gene of GI PBV (Rosen et al., 2000).

Before performing the RT reaction, 6 μL of extracted dsRNA and 2 μL of the primer PicoB25 (20 pmol) were incubated at 94 °C for 5 min (denaturation). Subsequently, it was placed on ice for 5 min. Then, were added 15 μL of RT mix containing 1x RT buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl; 15 mM $MgCl_2$, 50 mM dietilthreitol), 0.1 mM of each dNTP (EasyGen), 120 units of M-MLV Reverse Transcriptase (Promega) and DPEC water (Invitrogen) to a final volume of 25 μL. The mixture was incubated at 37 °C for 50 min and followed by inactivation at 70 °C for 5 min.

For amplification, 25 μL of the PCR mix consisting of 1x PCR buffer (20 mM Tris–HCl pH 8.4 and 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μL (20 pmol) of PicoB25 and 2 μL of PicoB45, 2.5 units Platinum Taq DNA polymerase (Invitrogen) and DPEC water (Invitrogen) were added to the RT reaction product to a final volume of 50 μL. The reaction was performed in a thermocycler (Bioneer) with the following time and temperature conditions: one step of 2 min/94 °C; followed by 40 cycles of 1 min/94 °C, 1 min/50 °C, 1 min/72 °C and a final step of 7 min/72 °C. The RT-PCR products were analyzed by 2% agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), pH 8.4, stained with ethidium bromide (0.5 μg/mL) and visualized under UV light.

2.6. Nucleotide sequencing and phylogenetic analysis

The RT-PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), quantified in a Qubit Fluorometer (Invitrogen) and sequenced in an ABI3500 Genetic Analyzer sequencer using a BigDye Terminator v3.1Cycle Sequencing Kit (Applied Biosystems).

Sequencing was performed in both directions using the forward (F) and reverse (R) primers corresponding to each PCR amplicon. The quality of each sequence obtained was analyzed with Phred software http://asparagin.cenargen.embrapa.br/phph/) and the sequence identity was verified with sequences deposited in the GenBank using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide (nt) identity matrix was constructed by BioEdit software version 7.1.3.0, and the phylogenetic tree based on the nt sequences was constructed by the neighbor-joining method and Kimura two-parameter model in MEGA software version 5.01 (Tamura et al., 2011). Bootstrapping was statistically supported with 1000 replicates.

2.7. Nucleotide sequence accession number

The sequence of bovine PBV strain obtained in this study was deposited in the GenBank database under accession number KP843617.

2.8. Statistical analysis

The chi-squared test was used at 5% significance to verify the differences among the proportions of positive or negative fecal

Table 1
Frequency of *Picobirnavirus* (PBV) detected by silver stained-polyacrylamide gel electrophoresis (ss-PAGE) from dairy calves fecal samples according fecal consistency.

Fecal consistency	PAGE results		
	Positive (%)	Negative (%)	Total
Diarrheic	18 (10.2)	158 (89.8)	176
Non-diarrheic	6 (5.3)	107 (94.7)	113
Total	24 (8.3)	265 (91.7)	289

$\chi 2 = 1.587$ $p = 0.2077$.

samples to PBV and fecal consistency (diarrheic/normal). The Epi-Info 6.0 program (Centers for Diseases Control and Prevention, Atlanta, USA) was used to perform the statistical calculations.

3. Results and discussion

From the 289 stool samples of individual calves analyzed by ss-PAGE the PBV was detected in 24 (8.3%) of them, which 10.2% (18/176) had diarrheic consistency (Table 1).

As like other studies, bovine PBV were found incidentally during bovine rotavirus research in diarrheic and non-diarrheic fecal samples. Although the prevalence of bovine PBV are still scarce in the literature, the percentage of 8.3% was much higher than that reported in other studies that also used ss-PAGE as diagnostic technique. Although many epidemiological studies involving human and porcine PBV has been described so far, there are only five reports describing the presence of PBV in feces of cattle or buffaloes of which one in Belgium (Vanopdenbosch and Wellemans, 1989), one in Brazil (Buzinaro et al., 2003) and three in India (Ghosh et al., 2009; Malik et al., 2011; 2013).

PBV was detected in only one diarrheic specimen of 78 samples tested from calves that had diarrhea in India (Ghosh et al., 2009). Another study also carried out in India found 3.67% (5/136) positivity for PBV, which four of these were from buffalo calves and one from cow calf, all of them without rotavirus infection (Malik et al., 2011).

In the only study of PBV detection in the Brazilian cattle population, Buzinaro et al. (2003) detected the PBV in four (0.76%) of 576 fecal samples evaluated (diarrheic and non-diarrheic) from calves less than 45 days old. All the 24 PBV positive calves identified in the present study were up to 60 days old, which 75% (18/24) had diarrhea at the moment of stool sampling. Statistical analysis using the chi-square test showed no significant difference ($p > 0.05$) between diarrheic and normal fecal samples (Table 1).

Based on epidemiological studies in humans, the role of PBV as causative agent of diarrhea is still controversial. Although some studies describe the presence of virus in stool samples from adults and children with diarrhea, the PBV etiological relationship with the diarrheal syndrome cannot be established, since it has also been reported the presence of this virus in asymptomatic hosts (Ganesh et al., 2010). The PBV is currently listed as emerging and opportunistic pathogens in cases of diarrhea, especially in immunocompromised individuals (Giordano et al., 1999; González et al., 1998; Grohmann et al., 1993; Martínez et al., 2003; Rosen et al., 2000). The PBV was found in 21% (17/84) of fecal samples from patients with diarrhea of unknown etiology, a significantly higher rate than uninfected patients with diarrhea of unknown etiology (van Leeuwen et al., 2010). Besides, after comparing PBV nucleotide sequences identified in humans and animals, it was also suggested the zoonotic potential of these viruses (Ganesh et al., 2010).

Although these fecal samples were negative for bovine rotavirus (data not shown), we cannot conclude that PBV was the primary agent of the diarrhea since they were not analyzed for other common enteropathogens, such as bovine coronavirus, *Escherichia coli and Eimeria* spp.

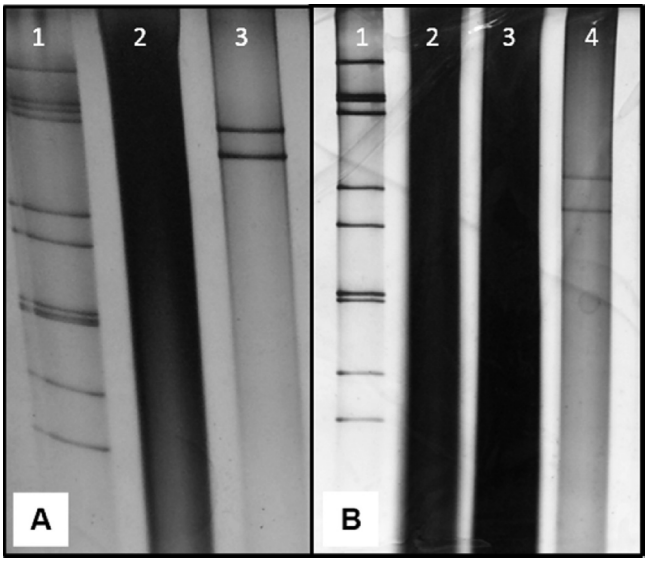

Fig. 1. *Picobirnavirus* (PBV) electrophoretic genomic profiles detected in dairy calves fecal samples by silver stained-polyacrylamide gel electrophoresis (ss-PAGE). (Panel A). Lanes 1: Genome pattern of specie A bovine rotavirus; lane 2: negative sample; lane 3: large genome profile of PBV. (Panel B). Lane 1: Genome pattern of specie A bovine rotavirus; lanes 2 and 3: negative samples; lane 4: small genome profile of PBV.

Table 2
Picobirnavirus (PBV) detected by silver stained-polyacrylamide gel electrophoresis (ss-PAGE) in dairy calves fecal samples according origin (municipality), fecal consistency and genome profile.

Municipality	Diarrheic			Non-diarrheic			
	PBV profile		Negative	PBV profile		Negative	Total
	Large	Small		Large	Small		
Cafelândia	5	3	40	4	0	47	99
Mariluz	4	1	39	1	0	25	70
Palotina	3	0	28	0	0	13	44
Toledo	1	0	27	0	0	11	39
Marechal C. Rondon	1	0	24	0	1	11	37
Total	14	4	158	5	1	107	289

According to the migration profile of the two dsRNA segments in ss-PAGE, both electrophoretic profiles of PBV were detected (Fig. 1). Of the 24 ss-PAGE positive stool samples, 5 (20.8%) of them showed a small electrophoretic profile and 19 (79.2%) samples had large profile. The distribution of small and large genome profiles of PBV according to stool consistency and origin (municipality) is shown in Table 2. The PBV has been detected in all five herds surveyed, having at least one positive animal. The large profile was detected in 79.2% (19/24) of the samples. This result is in agreement with others studies that describe this profile as the most frequent among PBV strains from different species hosts (Giordano et al., 2008; Malik et al., 2011; Masachessi et al., 2007). Surveys so far have only found PBV strains with the large RNA profile in bovine fecal samples (Buzinaro et al., 2003; Ghosh et al., 2009; Malik et al., 2011,2013; Vanopdenbosch and Wellemans, 1989). To the best of our knowledge, this is the first report of small genome profile PBV-like strains in naturally infected calves.

From the 24 ss-PAGE positive fecal samples, 15 (62.5%) were successfully amplified (201 bp) using GI specific primers targeting the RdRp gene of PBV. However, only large profile PBV samples were amplified using these primers (Table 3).

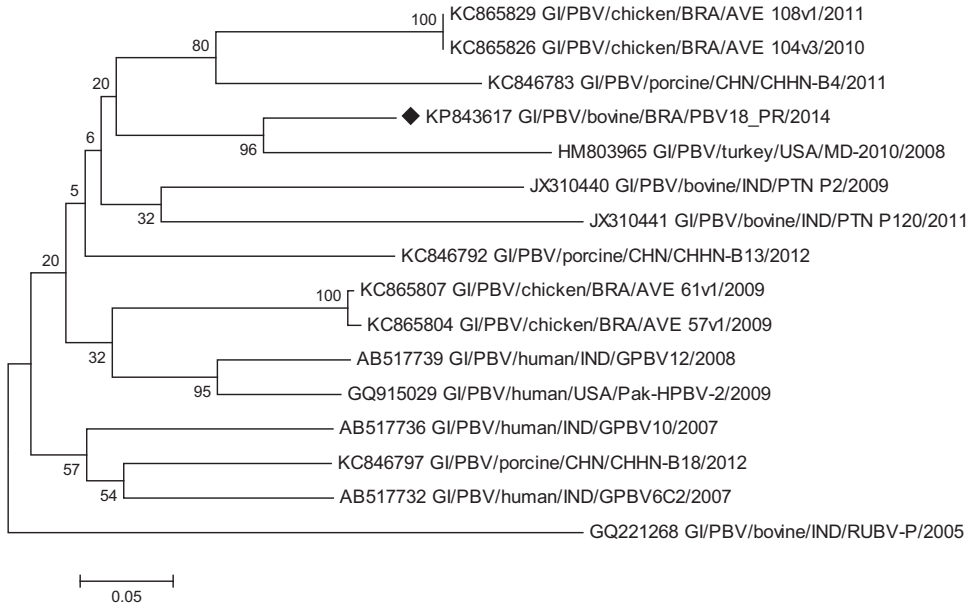

Fig. 2. Phylogenetic analysis among *Picobirnavirus* (PBV) genogroup I based on the partial fragment of the RdRp gene (segment 2). The bovine PBV sequence obtained in this study is indicated with the symbol "♦". The phylogenetic tree was constructed by using the Neighbor-Joining method and the Kimura 2-parameter nucletotide substitution. Bootstrapping was statistically supported with 1000 replicates. Scale bar indicates nucleotide substitutions per site.

Table 3
Picobirnavirus (PBV) detected by silver stained-polyacrylamide gel electrophoresis (ss-PAGE) and RT-PCR from dairy fecal samples according fecal consistency and genome profile. Only ss-PAGE PBV positive samples were evaluated by RT-PCR.

Fecal consistency	ss-PAGE and RT-PCR positive results for PBV					
	PBV large profile		PBV small profile		Total	
	ss-PAGE	RT-PCR	ss-PAGE	RT-PCR	ss-PAGE	RT-PCR
Diarrheic	14	11	4	0	18	11
Non-diarrheic	5	4	1	0	6	4
Total	19	15	5	0	24	15

This result is in agreement with Bhattacharya et al. (2006) that were only able to amplify large genome profiles of human PBV with the GI specific primers; the small genome profiles were amplified with GII specific primers.

As the small genome profile PBV-like strains, the remaining nine negative samples may suggest possible degradation of viral RNA, the presence of PCR inhibitors present in the stool or these samples may still belong to the GII PBV. However, since we have not tested the GII specific primer set it is not possible to assign to these PBV this classification. Bhattacharya et al. (2006), Fregolente el al. (2009), Martínez et al. (2003) also reported failure in the amplification of samples that were previously screened positive for PBV in ss-PAGE.

This is the first detection of bovine PBV by RT-PCR and molecular characterization in the American continent. Worldwide, there are only two molecular studies of PBV from cattle that were reported in India (Ghosh et al., 2009; Malik et al., 2014.). In Brazil, only one study involving PBV in bovine has limited to the viral genome detection by ss-PAGE (Buzinaro et al., 2003). On the other hand, other Brazilian studies were pioneers in the detection and molecular characterization of PBV in stool samples from rats, dogs and snakes (Fregolente et al., 2009) and more recently in broiler chickens (Ribeiro Silva et al., 2014).

Five amplicons were chosen randomly for sequencing and molecular characterization. The BLAST search analysis confirmed all sequenced amplicons as PBV. It was obtained a high similarity of these sequences with typical published PBV strains (data not shown). However, only one of these five sequences had quality enough to carry out the phylogenetic analysis.

Analysis of nucleotide identity matrix for the RdRp gene sequences revealed that GI/PBV/bovine/BRA/PBV18_PR/2014, identified in this study, showed the highest nucleotide identity (81%) with PBV strain detected in turkey (GenBank HM803965) and lowest identity (62.3%) with the prototype bovine PBV RUBV-P detected in India (GenBank GQ221268). This result was consistent with the phylogenetic analysis (Fig. 2); Bovine PBV18 grouped in the same phylogenetic branch of a PBV turkey, that have been discovered through metagenomics from turkey flocks experiencing enteric disease (Day et al., 2010).

Comparing with other sequences of bovine PBV (GenBank JX310440 and JX310441) nucleotide identities ranged from 66 to 71%. Interestingly, the genetic identity of bovine PBV strain, detected in this study, was higher with PBV strain heterologous species: broilers (72–75%), porcine (73%) and human (72%). Malik et al. (2014) have identified a bovine PBV strain (GenBank JX411964) which showed higher nucleotide identity (78.7%) with a human PBV (GenBank AJ504796) from a gastroenteritis outbreak in Hungary. However, similar to our study, this strain also revealed a high degree of divergence with the bovine PBV prototype RUBV-P.

Ghosh et al. (2009) have also reported the high genetic diversity of bovine PBV prototype strain, detected in diarrheic stool of 30 days-old calf. Comparing RUBV-P with PBV isolated from human and porcine hosts, nucleotide identity ranged from 51.2 to 64.9% and 53.5 to 64.1%, respectively. The identities with PBV detected in dogs, rats, and snakes were from 57.3 to 59.4%, 55.3%, and 52 to 60.6%, respectively. However, until this report was published, there were none sequence of bovine PBV to determine the genetic relatedness among isolates of the same species. Recently, a low degree of identity between RdRp gene fragment of PBV from a buffalo calf and the prototype bovine PBV strain RUBV-P was described for both nucleotide (23.5%) and amino acid (45%) levels (Malik et al., 2013).

Molecular characterization of bovine PBV RdRp gene revealed a high degree of genetic diversity even in PBV strains involving the same animal species, since the nucleotide sequences of PBV strains of bovine origin presented low identity between them. This

heterogeneity can be explained by genetic material of PBV, a segmented double stranded RNA that can facilitate the emergence of new strains by genomic rearrangements or reassortments (Parrish et al., 2008; Nates et al., 2011).

4. Conclusions

This study was conducted with the aim to report the occurrence of PBV in cattle population in dairy cattle from Paraná State, South Brazil and stablish phylogenetic relatedness with others PBV from different animal species. Although the both genomic profile of PBV (small and large) were found by ss-PAGE, only the large profiles were successfully amplified by RT-PCR using the primer pair PicoB25 and PicoB43 (Rosen et al., 2000). The most detected PBV in diarrheic samples was the large genome profile. It suggests that there may be a close relationship between large genome profile of PBV and diarrhea in calves.

Although there are available several partial sequences of RdRp gene PBV strains from various animal species and humans, it is still limited the number of the whole sequences in public databases that would allow for a better molecular characterization of these highly divergent virus. Further studies are needed to understand the relationship of high genetic variability in the epidemiology and evolution of PBV in animals and humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2015.09.022.

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