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REGULAR ARTICLES

Prevalence and molecular characterization of porcine *Picobirnavirus* in piglets of North East Region of India

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Abstract Picobirnaviruses (PBVs) have been recognized as one of the important causal viral agents of gastroenteritis in several animal species especially in young immunocompromised hosts. In this study, we report the prevalence and molecular epidemiology of porcine PBVs from North Eastern Hilly region of India. A total of 457 fecal samples from piglets were collected from local ($n = 130$) and cross ($n = 327$) breed piglets in different seasons for 2 years. All the samples were subjected to RNA-PAGE and RT-PCR analysis for detection of PBVs. A total of 4.59 and 11.15% samples were recorded as positive for PBVs by RNA-PAGE and RT-PCR, respectively. Rate of detection was higher from diarrhoeic animals (13.56%) compared to non-diarrhoeic (4.23%) animals. Higher prevalence rate was observed from unorganized farms (14.22%) compared to organized farms (8.0%) with slightly higher detection from cross breed (11.62%) compared to local breed (10.0%). Maximum cases of piglet diarrhea associated with PBVs were detected during summer (16.4%) and winter (14.39%) seasons compared to autumn (4.80%) and spring (6.45%). All the samples were positive for PBV genogroup I only. Based upon the sequence analysis, the isolates were unique and placed in separate clad and were not closely associated with any other Indian isolates of PBVs so far. Two isolates were closely related with one Chinese isolate recovered from sewage water. This is the first systematic study of prevalence of PBVs associated with piglet diarrhea.

Keywords *Picobirnavirus* · Prevalence · Piglets · India

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Introduction

The genus *Picobirnavirus* (PBV) is the only genus belonging to the family *Picobirnaviridae* under the proposed order *Diplornavirales* (Ganesh et al. 2014) that were first identified in children by Pereira et al. (1988a) from Brazil. Thereafter, PBVs were detected in fecal specimens of humans and wide range of animals, birds, rodents from different parts of the world (Malik et al. 2014). PBVs are regarded as enteric viruses as most of their detection is associated with virus shed in feces. They have been detected in animals and human patients with and without gastroenteritis and were found co-infected with other enteric viruses such as *Rotavirus*, *Astrovirus*, *Calicivirus*, and *Coronavirus* (Malik et al. 2014). PBVs genogroups I and II have been reported from respiratory tract specimens from pigs obtained in slaughter houses in China, Hongkong, and Sri Lanka (Smits et al. 2011) and from environmental samples including community sewage and surface waters, which indicate their worldwide prevalence in nature (Symonds et al. 2009; Hamza et al. 2011). In India, only a handful of information on PBV infection in bovines (Malik et al. 2011) and domestic pigs (Ganesh et al. 2012) are

available, but no systematic study on prevalence of PBV infection in domestic animals has been conducted so far.

The North East Hilly Region of India is mainly inhabited by tribal population, where pig farming is an integral part of life and significant source of income with the highest pig population (3.95 million) in India (19th Livestock census 2012). Porcine viral gastroenteritis is one of the most common diseases affecting the piggery industry in this region. In the present study, an attempt had been made to understand the prevalence of PBVs in piglets (below 3 months of age) with or without diarrhea and molecular characterization of porcine PBVs in the North Eastern Hilly Region of India. This seems to be the first report of its kind.

Materials and methods

Collection of fecal samples

A total of 457 fresh fecal samples were collected from piglets (<3 months) from organized ($n = 225$) and unorganized ($n = 232$) farms of four North Eastern Hilly states of India, viz., Manipur ($n = 108$), Meghalaya ($n = 124$), Mizoram ($n = 120$), and Nagaland ($n = 105$). Samples were collected from diarrhoeic ($n = 339$) and non-diarrhoeic ($n = 118$) piglets including indigenous local ($n = 130$) and cross breed (LWY × local germ plasm) ($n = 327$) piglets (Table 1). Samples were collected in four different seasons of the year, viz., spring (March–May) ($n = 93$), summer (June–August) ($n = 128$), autumn (September–November) ($n = 104$), and winter (December–February) ($n = 132$) during June, 2013 to May, 2015.

Sample processing and extraction of viral nucleic acids

A 10% suspension of each fecal sample was prepared with phosphate buffered saline (PBS; pH 7.4) by dissolving 0.1 g of feces in 1 ml PBS. The suspension was vortexed for 2 min followed by centrifugation at 10,000 rpm for 20 min at 4°C to remove the course debris. The clarified supernatant was collected and stored at 4°C for short-term storage and −20 or −80°C for longer term. RNA was extracted from supernatant of fecal samples by Trizol extraction method as per the WHO Manual (2009). Quality, quantity, and purity of the total RNA were checked in a Nanodrop Spectrophotometer (Thermo Scientific, USA).

Detection of dsRNA of *Picobirnavirus* by RNA-PAGE

The electrophoresis of viral RNA was carried out (Malik et al. 2011) in 10% native (non-denaturing) polyacrylamide gel in Tris–Glycine buffer (0.025 M Tris,0.109 M Glycine, pH 8.3) by loading up to 500 ng of viral RNA per well. The viral genomic electrophoresis was also carried out in denaturing 5% polyacrylamide gel (containing 7 M Urea) in 1 × TBE running buffer (8.9 mM Tris, 8.9 mM Boric acid, 0.2 mM EDTA, pH 8.3) by loading the same amount of viral RNA per well. Samples were electrophorosed at 100 V until the dye reached to be the end of the gel (approx. 4 h). The gel was silver impregnated following the method of Herring et al. (1982) and documented. For the estimation of molecular weight of the two segments of PBV, samples were run with group A rotavirus and 1 kbps DNA ladder (Fermentas) on 1× agarose gel, stained with ethidium bromide and documented in Gel doc system (Alpha Image, USA). The dsRNA nature of these segments was confirmed by nuclease treatment.

Table 1 Prevalence of *Picobirnavirus* detected by RNA-PAGE from fecal samples of piglets of the four NEH states of India

State	Positive samples		Diarrhoeic	Non-diarrhoeic	Local breed	Cross breed
Manipur	Organized	1/46 (2.17%)	1/32 (3.13%)	0/14	0/4	1/42 (2.38%)
	Unorganized	2/62 (3.23%)	2/46 (4.35%)	0/16	0/24	2/38 (5.26%)
Subtotal		3/108 (2.77%)	3/78 (3.84%)	0/30	0/28	3/80 (3.75%)
Meghalaya	Organized	2/66 (3.03%)	2/48 (4.17%)	0/18	0/12	2/54 (3.70%)
	Unorganized	7/58 (12.07%)	6/42 (14.29%)	1/16 (6.25%)	2/22 (9.09%)	5/36 (13.89%)
Subtotal		9/124 (7.25%)	8/90 (8.88%)	1/34 (2.94%)	2/34 (5.88%)	7/90 (7.77%)
Mizoram	Organized	2/57 (3.51%)	2/45 (4.44%)	0/12	0/14	2/43 (4.65%)
	Unorganized	5/63 (7.94%)	4/52 (7.69%)	1/11 (9.09%)	2/26 (7.69%)	3/37 (8.11%)
Subtotal	7/120 (5.83%)	6/97 (6.18%)	1/23 (4.34%)	2/40 (5.0%)	5/80 (6.25%)	
Nagaland	Organized	1/56 (1.79%)	1/38 (2.63%)	0/18	0/11	1/45 (2.22%)
	Unorganized	1/49 (2.04%)	1/36 (2.78%)	0/13	0/17	1/32 (3.13%)
Subtotal		2/105 (1.90%)	2/74 (2.70%)	0/31 (0.0%)	0/28 (0.0%)	2/77 (2.59%)
Grand total		21/457 (4.59%)	19/339 (5.60%)	2/118 (1.69%)	5/130 (3.84%)	16/327 (4.89%)

Digestion of nucleic acid was performed following the method described by Pereira et al. (1988b). Briefly, 1 μg of purified PBV RNA was treated separately with pancreatic RNase A, RNaseT1, and DNase 1. The pancreatic RNase A was used after boiling for 10 min at a final concentration of 10 ug/ml in Tris–EDTA–NaCl (10 mM Tris–HCl, 10 mM NaCl, 2 mM EDTA), while RNaseT1 was used at a final concentration of 10 U/ml in the same buffer mentioned above and DNase1 was used at 10 U/ml in 40 mM Tris–HC1 (pH 7.9), 10 mM $CaC1_2$, and 6 mM $MgC1_2$. Digestion was carried out at 37°C for 30 min, and following GIT extraction and isopropanol precipitation, the products were analyzed on 10% non-denaturing and 5% denaturing polyacrylamide gel.

Detection of *Picobirnavirus* by RT-PCR

Characterization of PBVs was done by RT-PCR targeting gene segment 2 (RdRp gene) for PBV genogroup I (Rosen et al. 2000) and PBV genogroup II (Smits et al. 2011). The PCR were conducted in a thin-walled PCR tube in total volume of 25 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, 20pM of each primers, and 3.0 μl of previously synthesized cDNA. The PCR was performed in a thermal cycler (Mastercycler Gradient, Eppendorf, Germany) in a cyclic condition: initial denaturation at 940°C for 5 min, followed by 36 cycles of denaturation at 94°C for 3 min, annealing at 48°C for PBV GGI and 56°C for PBV GG II for 20 s, 72°C for 30 s, and final extension at 72°C for 8 min. The amplicons were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in Tris–borate buffer and were visualized with a UV transilluminator and photographed by gel documentation system (AlphaImager, USA).

Cloning, sequencing, and sequence analysis

Extracted PCR products were purified (QIAGEN kit) and cloned in TA cloning vector (MBI Fermentas) and sent for sequencing to DNA sequencing facility, Department of Biochemistry, University of Delhi, South Campus, New Delhi, using automated sequencer Excel Applied Biosystem 3730 (USA). Sequencing data were analyzed using MegAlign program. Phylogenetic and bootstrap analyses were performed using neighbor joining and seqboot programs. Partial nucleotide sequences of isolates of the present study have been deposited in NCBI GenBank (Accession nos. KT032190, KT380848, KT380849, and KT380850).

Results

Prevalence of *Picobirnavirus* in piglets by RNA-PAGE

The overall prevalence of PBVs in piglets by RNA-PAGE was recorded as 4.59% (21/457). Meghalaya showed the highest (7.25%) prevalence followed by Mizoram (5.83%), Manipur (2.77%), and Nagaland (1.9%). Prevalence of PBVs was recorded higher in unorganized farms (6.46%) compared to the organized farms (2.66%). Similarly, rate of detection of PBVs from diarrhoeic animals was higher (5.60%) than that from non-diarrhoeic animals (1.69%) in all the states throughout the study period. The breed-wise analysis revealed that cross breed animals were more susceptible (4.89%) than the local breed of animals (3.84%) (Table 1).

Prevalence of *Picobirnavirus* in piglets by RT-PCR

The similar prevalence study was also recorded by RT-PCR. Detail of the result is depicted in Table 2. The overall

Table 2 Prevalence of *Picobirnavirus* detected by RT-PCR from fecal samples of piglets of the four NE states of India

State	Positive samples		Diarrhoeic	Non-diarrhoeic	Local breed	Cross breed
Manipur	Organized	4/46 (8.70%)	4/32 (12.50%)	0/14	0/4	4/42 (9.52%)
	Unorganized	8/62 (12.90%)	7/46 (15.22%)	1/16 (6.25%)	3/24 (12.50%)	5/38 (13.16%)
	Total	12/108 (11.11%)	11/78 (14.10%)	1/30 (3.33%)	3/28 (10.71%)	9/80 (11.25%)
Meghalaya	Organized	8/66 (12.12%)	7/48 (14.58%)	1/18 (5.56%)	1/12 (8.33%)	7/54 (12.96%)
	Unorganized	12/58 (20.69%)	10/42 (23.81%)	2/16 (12.50%)	4/22 (18.18%)	8/36 (22.22%)
	Total	20/124 (16.12%)	17/90 (18.88%)	3/34 (8.82%)	5/34 (14.70%)	15/90 (16.66%)
Mizoram	Organized	5/57 (8.77%)	5/45 (11.11%)	0/12	1/14 (7.14%)	4/43 (9.30%)
	Unorganized	10/63 (15.87%)	9/52 (17.31%)	1/11 (9.09%)	3/26 (11.54%)	7/37 (18.92%)
	Total	15/120 (12.5%)	14/97 (14.43%)	1/23 (4.34%)	4/40 (10.0%)	11/80 (13.75%)
Nagaland	Organized	1/56 (1.79%)	2/38 (5.26%)	0/18	0/11	1/45 (2.22%)
	Unorganized	3/49 (6.12%)	2/36 (5.56%)	0/13	1/17 (5.88%)	2/32 (6.25%)
	Total	4/105 (3.80%)	4/74 (5.40%)	0/31 (0.0%)	1/28 (3.57%)	3/77 (3.89%)
Grand total		51/457 (11.15%)	46/339 (13.56%)	5/118 (4.23%)	13/130 (10.0%)	38/327 (11.62%)

prevalence of PBVs based on RT-PCR was much higher (11.15%, 51/457) than RNA-PAGE (4.59%, 21/457) analysis. All the positive samples were found to be under *Picobirnavirus* genogroup 1 only. Meghalaya showed the highest (16.12%) prevalence followed by Mizoram (12.50%), Manipur (11.11%), and Nagaland (3.80%). The unorganized farms showed higher prevalence (13.56%) compared to the organized farms (8.00%). The rate of detection of PBVs from diarrhoeic animals was higher (13.56%) than that from non-diarrhoeic animals (4.23%) in all the states throughout the study period. The breed-wise analysis also exhibited that cross breed animals were more susceptible (11.62%) than the local breed of animals (10.00%).

Sequencing and phylogenetic analysis of the isolates

Representative samples from each state (Meghalaya, Manipur, Mizoram and Nagaland) of NEH region of India were selected for nucleotide sequencing (RdRp gene region) (GenBank accession no. KT032190, KT380848, KT380849, and KT380850). Selected four isolates under this study are appeared in three different clads (Fig. 1). Two isolates, one each from Manipur (GenBank accession no. KT380848) and Mizoram (GenBank accession no. KT380849), are located in the same unique clad with 100% identity between them. Both the isolates are also placed with one human isolates from India. One isolate from Nagaland (NER-3NL-PBV-Ind; GenBank accession no. KT380850) is also distinctly placed in the phylogenetic tree and not associated with any of the Indian isolates. Isolate no. PPBV/ML/Ind/U-1 (GenBank accession no. KT032190) from Meghalaya is clustered with Chinese PBV isolated from sewage but placed distinctly.

Season-wise prevalence of *Picobirnavirus* in North Eastern Hilly Region of India

Prevalence of *Picobirnavirus* in piglets of NEH region of India is depicted in Table 3. Prevalence was significantly high during summer (16.40%) and winter (14.39%) compared to spring (6.45%) and autumn (4.80%).

Discussion

As depicted in Tables 1 and 2, prevalence of PBVs in young piglets in the NEH region of India is 11.15%. The difference in prevalence by RNA-PAGE and RT-PCR is well established being the RNA-PGE is less sensitive technique in comparison to RT-PCR for detection of RNA virus infection in host (Malik et al. 2014). Majority of the earlier worker in different parts of world recorded higher prevalence of PBVs in man and/or animals (Carruyo et al. 2008; Banyai et al. 2008; Wang et al. 2007, 2012) by RT-PCR than RNA-PAGE. But in contrast to them, few workers were also recorded either equal (Ganesh et al. 2012) or less prevalence of PBVs (Fregolente et al. 2009) by RT-PCR than RNA-PAGE. Prevalence of PBVs in pigs is highly variable (0.4 to 65%) globally (Malik et al. 2014). In India, only one report on detection of *Picobirnavirus* in pigs from Kolkata is available (Ganesh et al. 2012) with a limited number of sample size ($n = 11$), of which 18.20% were positive by both RNA-PAGE and RT-PCR.

All the positive samples under the present study exhibited the presence of GG1 *Picobirnavirus* in the piglets of NEH region of India. Ganesh et al. (2012) also detected only GG1 PBV from pigs of Kolkata, India. Although, GGII PBV is reported from India in other animals (Malik et al. 2014) and human (Ganesh et al. 2010) hosts but not from pigs. More systematic studies need to be conducted to establish the prevalence of genogroups of PBV in pigs in India.

In the present study, rate of incidence of PBVs in diarrhoeic animals (13.56%) is much higher than the non-diarrhoeic animals (4.23%) (Tables 1 and 2). Gatti et al. (1989) tried to establish the relation of PBVs with diarrhea in animals by screening 912 fecal samples of pigs in Brazil. They could recover PBVs alone or as mixed infection with rotavirus in 15.30% diarrhoeic and 9.60%

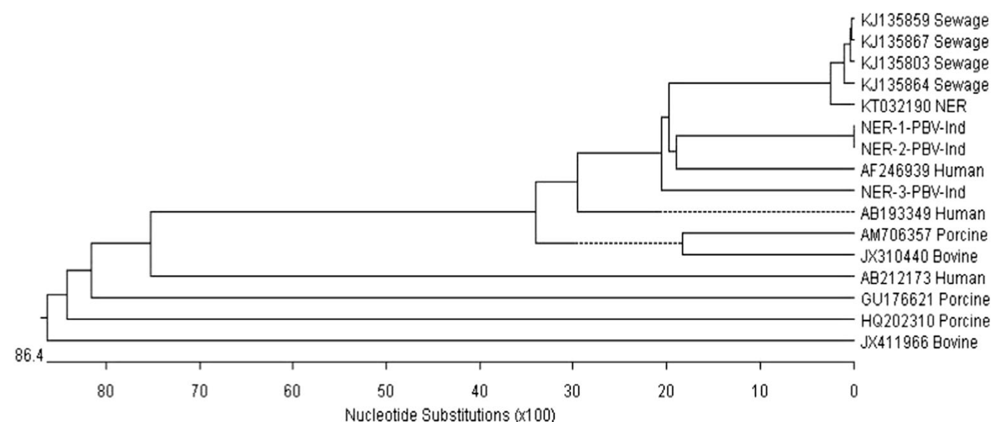

Fig. 1 Phylogenetic tree showing genetic relatedness between the *Picobirnavirus* isolated from piglets of four North Eastern hilly states of India and other *Picobirnavirus* isolates from man, animals, and environment of India and other countries based upon the nucleotide sequences of RdRp gene region of GG1 *Picobirnavirus*

Table 3 Season-wise prevalence of *Picobirnavirus* detected from fecal samples of piglets of the four NEH states of India

Sl. No.	Season	No. of samples screened	No. of samples positive (%)
1	Spring (March–May)	93	6 (6.45%)
2	Summer (June–August)	128	21 (16.40%)
3	Autumn (September–November)	104	5 (4.80%)
4	Winter (December–February)	132	19 (14.39%)
	Total	457	51 (11.15%)

$\chi^2 = 11.258$, $^{**}\chi^2_{12} = 4.977$, $^{*}\chi^2_{13} = 0.252$, $\chi^2_{14} = 3.485$, $\chi^2_{23} = 7.757$,$^{**}\chi^2_{24} = 0.202$, $^{**}\chi^2_{34} = 5.852^{*}$

*Significantly different ($p < 0.05$), **Significantly different ($p < 0.01$)

non-diarrhoeic pigs. But Ludert et al. (1991) failed to establish any such conclusion, where high incidence of PBV infection without diarrhea (12.30%) compared to pigs with clinical diarrhea (10.0%) is reported. Picobirnavirus is not considered to be as a primary pathogen associated with diarrhea in man and animals rather an opportunistic organisms can develop the clinical disease in association with other established pathogens, viz., rotavirus, astrovirus, coronavirus, *E. coli*, *Salmonella*, etc., (Malik et al. 2014). But recovery of high level of PBV from the diarrhoeic piglets under the present study has definitely indicated its role in the development of diarrhea in the host. In India, prior to this report, no such study has been undertaken in this regard so far.

Prevalence of diarrhea associated with PBV is recorded higher in unorganized farms (13.56%) compared to the organized farms (8.00%). The result may have a direct correlation with the managemental practices. Picobirnaviruses can directly enter and transmit through sewage water in the farms (Symonds et al. 2009). The small and landless farmers with a handful of animals are not able to maintain proper hygienic practices at their own which may be the reason to attract the PBV in the farm. Prevalence of PBVs in cross breed animals is recorded as higher (11.62%) than local animal population (10.0%). Although the variation is not very wide or significant, but it may be possible that the local non-descriptive animals possess better protective nature against natural infection than the exotic or cross breed animals. In addition, the weaning age of piglets of local animals is generally 8–10 weeks in comparison with the cross breed animals in organized farms (within 6 weeks). Maternal immunity might also play an important role in resisting the infection in piglets. The close proximity of the cross bred animals reared in the commercial farms may also be one of the reasons for this fact.

In the present study, for the first time, we have tried to record the seasonal variation of PBV infection in piglets. Interestingly, we have recorded a wide variation between summer (16.40%) and winter (14.39%) with spring (6.45%) and autumn (4.80%). To the best of our knowledge, no such studies are conducted to record the seasonal effect on PBV infection in piglets. We have collected the samples consistently for 2 years from the similar location with similar frequency. The humid climatic condition during summer and winter with persistent rain fall in this region of country may be an influential factor for persistence and consistent spread of infection among the animals. The similar climatic condition is also a favorable environment for other enteric pathogens like *E. coli*, *Salmonella*, rotavirus, coronovirus which are considered to be the common associated pathogens of PBV for the development of diarrhea in piglets. Studies describing the seasonal pattern of PBVs in diarrhoeic piglets or other animal and in fact in human have rarely been published. Paucity of literatures regarding this did not allow us to compare our results.

PBVs are genetically diverse and are evolving rapidly (Ganesh et al. 2012). Although obtained from same host and within a small geographical area, selected four isolates under this study were appeared in three different clades indicating their genetic diversity. Interestingly, two isolates (GenBank accession no. KT380848 and KT380849) that are located in same unique clade with 100% sequence homology are also placed with one human isolates from India (Fig. 1). This result strongly suggests that PBV strains may circulate in the shared environment, where human and animals survive. In addition, all the four isolates were distinctly placed in the phylogenetic tree and are not associated with the genomic sequence of any of the other Indian isolates from human or animals. So to know the possible origin of the PBVs recorded in the piglets in NEH region of India needs to be studied in detail under the future study. Transmission of PBVs from animals to man or man to animals is not yet established. Although the sequence similarities are obtained using partial sequence of a single gene region (RdRp), but detection of PBVs in sewage and surface waters at relatively high frequency and the close proximity of animals and man as well as sequence similarities between the virus isolates obtained from them are important indicators of its possibilities.

In conclusion, it may be stated that PBVs are persistently associated with the piglet diarrhea in NEH region of India. Prevalence of PBVs is high in summer and winter seasons compared to the autumn and spring seasons with higher prevalence recorded in cross breed pigs compared to the local pigs. Based upon the sequence analysis, the isolates are unique and placed in separate clade and are not closely associated with any other Indian isolates of PBVs so far. Two isolates were closely related with one Chinese isolate recovered from sewage water. This is the first systematic study of prevalence of PBVs associated with piglet diarrhea.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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