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PORCINE PICOBIRNAVIRUS INFECTION IN VENEZUELAN FARMS

Infección por Picobirnavirus Porcino en Granjas Venezolanas

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ABSTRACT

Detection frecuency of picobirnaviruses (PBV) genome was evaluated by polyacrylamide gel electrophoresis (PAGE) in 402 piglets faecal samples collected in eight farms from the Central Region of Venezuela. Electrophoretic variability of the genome profile was also evaluated. The presence of PBV and Rotavirus was observed in 50 (12%) and 51 (13%) of the samples, respectively. However, at variance with Rotaviruses, PBV was equally distributed among samples from diarrhoeic and non-diarrhoeic animals. Analysis by electron microscopy of selected positive samples after cesium chloride isopicnic centrifugation confirmed the presence of PBV. PBV were found in all age groups tested (7 to 56 days old), and genetic variability was observed among isolates. The genome analysis by PAGE of differents PBV isolates revealed slight variations in the migration patterns for genomic segments 1 and 2. The presence of double or multiple bands in the position corresponding to each genomic segment was also observed, suggesting the coinfection of a single animal with different PBV isolates. Most of the ARN genome segments showed a wide electrophoretic pattern, larger and smaller genome segments ranging from 2.5 to 2.3 and 1.7 and 1.8 kpb, respectively. Narrow electrophoretic profiles were also observed where both bands were located between rotavirus Ohio State University strain (OSU) segments 4 and 5. Analysis by Reverse Transcription and Polimerase Chain Reaction (RT-PCR) indicated that these samples were indeed positive for PBV. These results confirm that genetically variable PBV strains circulate frequently in piglets, but their pathogenic potential remains elusive.

Key words: PBV, rotavirus, diarrhoea, dsRNA, piglets.

RESUMEN

La presencia de Picobirnavirus (PBV) se evaluó a través de electroforesis en geles de poliacrilamida (EGPA) a partir de 402 muestras fecales de lechones recolectadas en ocho granjas de la Región Central de Venezuela. Evaluada la variabilidad electroforética observada en sus dos segmentos genómicos. La presencia de PBV y Rotavirus se observó en 50 (12%) y 51 (13%) muestras. Sin embargo, a diferencia de Rotavirus, la detección de PBV se observó igualmente distribuida, tanto en animales con diarrea como sin diarrea. La presencia de PBV fue confirmada por microscopía electrónica. PBV fue detectado en todos los grupos etarios muestreados (desde 7 a 56 días de edad) y se observó variabilidad genética entre los aislados. El análisis por EGPA del genoma de diferentes aislados de PBV reveló ligeras variaciones en el patrón de migración de sus dos segmentos genómicos 1 y 2. Asimismo, se observó la presencia de doble o múltiples bandas en la posición correspondiente a cada segmento genómico, lo cual sugiere la coinfección de un animal con diferentes variantes de PBV. La mayoría de los segmentos de ARN genómico aislados mostraron un patrón electroforético amplio, cuyos tamaños comprendían entre 2,5 a 2,3 y 1,7 a 1,8 kpb, para los segmentos 1 y 2, respectivamente. También se observaron perfiles electroforéticos estrechos, en los que las dos bandas se ubicaron entre los segmentos 4 y 5 de rotavirus cepa Ohio State University (OSU). Estas muestras resultaron positivas a PBV por Transcripción Reversa-reacción en cadena de la Polimerasa (RT-PCR). Estos resultados confirman la circulación frecuente de varias cepas de PBV en lechones, pero su potencial patógeno aún no se ha definido.

Palabras clave: PBV, rotavirus, diarrea, ARNdc, lechones.

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INTRODUCTION

The picobirnaviruses (PBV) are a group of viruses belonging to the newly proposed virus family, *Picobirnaviridae* under the order *Diplornavirales* [3, 13], PBV were accidentally discovered in Brazil by Pereira and collaborators while analyzing fecal samples from free living rats (*Oryzomys nigripes*) [33]. Since then, the presence of PBV have been reported in the feces of a large number of vertebrate species, including humans [8, 11, 14, 19, 22, 26, 31, 32], non human primates [43], pigs [9,17, 25, 34, 35], chickens [1, 24, 30, 40], rabbits [15, 27] foals [5], dogs [10] and wild animals kept in captivity [23, 28, 29]. Recently, the widespread presence of PBV in waste waters was reported [39].

The PBV genome consist of two segments of double stranded RNA (dsRNA) whose sizes range from 2,120-2.600 pb and 1,330-1.900 pb for the segment 1 or 2, respectively [14, 15, 17, 25, 41]. Two open reading frames (ORF) have been identified in genomic segment 1; the second ORF encodes for the coat protein but the protein encoded by the ORF 1 is yet unknown has not been established alredy. The genomic segment 2 encodes for a protein of 534 amino acids which shows domains typical of RNA dependent RNA polymerases [7, 12, 36, 41]. The 35nm non enveloped viral particle shows to have a single-layered coat protein with a unique architecture made by 120 proteins subunits, displaying 60 two-fold symetric dimers with a new 3D-fold [12, 25].

Porcine PBV were first reported by Gatti *et al.* [17] who found a prevalence of 11.6% in fecal samples collected in farms of the Sao Paulo State, Brazil, using visualization of the PBV genome by Polyacrylamide Gel Electrophoresis (PAGE) and silver staining as detection method. Very similar prevalences were reported shortly afterwards by Ludert *et al.* [25] using the same technique to analyze samples collected in Venezuelan farms. PBV have also been observed in porcine fecal samples collected from piglets in the United Kingdom (UK), Thailand and Mexico, albeit with a lower frequency (> 2%) [9, 34, 35].

Despite the numerous reports of the presence of PBV in fecal samples from vertebrates, the pathogenicity of these viruses has not been established. For example, contradictory results had been obtained regarding the association of PBV with diarrhoea in pigs [17, 25] and asymptomatic excretion has been frequently reported from humans and animals [6, 24, 28]. On the other hand, studies conducted in Human Immunodificience Virus (HIV)-infected people [18, 20, 22] suggest that PBV may be an opportunistic pathogen that may cause diarrhoea in individuals with immunosuppression.

In young animals of economic importance such as pigs (*Sus scrofa domesticus*), diarrhoea constitutes a major cause of low feed conversion, resulting in diminished gain or loss of weight and stunting. So far it has not been possible to establish clearly the disease burden, if any, that PBV infections may cause in the diarrhoea processes suffered by their hosts. On the other hand, it is clear the wide distribution and genetic variability of porcine PBV in nature [2, 7]. Thus, the main objectives of this work were to detect the presence of PBV in fecal samples using PAGE as a detection method, to analyze the heterogeneity of the genomic electrophoretic patterns and to revaluate the possible association between PBV excretion and the presence of diarrhoea in piglets.

MATERIALS AND METHODS

Farms

Eight farms from Aragua, Miranda, Carabobo, Yaracuy and Guarico States located en Central Region of Venezuela were sampled during the present study. Santiago Mariño and Urdaneta, Sucre, Diego Ibarra, Peña and Juan Germán Roscio, Municipalities, respectively.

Faecal samples

A total of 402 fecal samples from piglets were collected in eight farms located in the Central Region of Venezuela. Samples were collected from piglets 7 to 56 days (d) old since the post-weaning period in the sampled farms usually start around d 21. Samples were taken directly from the floor of the cage immediately after defecation, transported on wet ice to the laboratory and kept at -70°C (Revco ULT-2186-3A, Thermo Electron Corporation, USA) until processing. According to the consistency and veterinary clinical judgment, faeces were classified as diarrhoeal and non-diarrhoeal.

Extraction of RNA and analysis by polyacrylamide gel electrophoresis

Fecal suspensions (10% w/v) were prepared in Eppendorf 1.5 mL. tubes, using equal parts of bidistilled water and freon (1,1,2-trichloro-1 ,2,2-trifluoroethane, DuPont, Wilmington, USA). The suspensions were homogenized by strong agitation for 10 minutes (mn) in a vortex (Analog vortex mixer/ VWR™ International, USA) and clarified by centrifugation at 10000 xg for 5 min at room temperature in a microcentrifuge Eppendorf model 5415C, USA. Aliquots of 250 µL of each clarified fecal sample were used for total RNA extraction using TRIzol® LS (Life Technologies™), according to the protocol suggested by the manufacturer. Finally, the pellet was dried, resuspended in 20 µL of bidistilled sterile water and stored at -70°C until use. The RNA extracted was analyzed by 7% polyacrylamide gel electrophoresis [26].

After the electrophoretic run, genomic nucleic acids were visualized by silver staining as previously described [25].

The genome of the porcine rotavirus OSU strain was used as a reference and positive control during the electrophoretic runs. The genome of rotavirus consists of 11 segments of dsRNA whose molecular weight and migration pattern is well known.

Virus purification

Virus purification was carried out as described by Ludert *et al.* [25]. Briefly, clarified fecal suspensions (20% w/v) were prepared from samples that tested positive only to PBV by PAGE and further centrifuged at 7500 rpm to 7°C for 20 min in a centrifuge Sorvall RC-5 (SuperSpeed Refrigerated Centrifuge, Dupont Instruments, USA) to pellet bacteria and cell debris. Viruses were concentrated through a cushion of 40% sucrose in phosphate buffer saline (PBS) by ultracentrifigation at 110.000xg for 3 hours (h) to 7°C in a rotor SW28 (Beckman Instruments, Inc., USA). The pellet obtained was resuspended 0.5 mL PBS. Finally, virus preparations were concentrated and purified by flotation in an isopicnic CsCl gradient by ultracentrifugation in a SW41 rotor at 110.000xg for 48h. Fractions (0.5 mL) were collected from the bottom of the tube and their refractive index measured. Fractions with a refractive index between 1.3720 and 1.3745, corresponding to densities between 1406 gr / mL and 1,435 g / mL [21], were pooled and virus particles pelleted by further centrifugation at 110.000xg for 3 h at 4°C in a SW 41 rotor. The sediment obtained was resuspended in 250 µL PBS and stored at -70°C.

Electron microscopy

To demonstrate the presence of viral particles, purified viral preparations were checked directly under the electron microscope (Phillips CM10, Eindhoven, The Netherlands). Samples were negative strained with 2% phosphotungstic acid for 1 min. The viral particles were measured and identified as PBV according to their morphology.

Statistical analysis

Statistical analysis to establish differences in the frequency of PBV detection among samples from animals with and without diarrhoea, and among the different age groups were made using the chi square test and the Epi-Info program (version 2.1. CDC, Atlanta, GA).

RESULTS AND DISCUSSION

Detection of porcine PBV

In order to determine the presence of PBV in stool from piglets, all samples were analyzed by PAGE after nucleic acid extraction (FIG. 1). A sample was considered positive when the presence of the two electrophoretic bands, corresponding to the PBV genome, was detected. PBV was detected in 50 (12%) samples. Three percent of the samples yielded inconclusive results for PBV due to the presence of single bands or atypical patterns. In addition, 51 (13%) samples were found positive for rotaviruses. Mixed infections by rotavirus and PBV was seen in 10 (2%) of samples collected.

Twenty five percent of the samples (101/402) were classified as “diarrhoea” according to their consistency, and the rest (301) as “non-diarrhoea”. No statistical significant difference was observed in the frequency of PBV detection between samples classified as diarrhoea or non-diarrhoea (7 versus 14%, P= 0.05260). In contrast, the presence of rotaviruses was significantly higher in the “diarrhoea” samples than in the “no- diarrhoea” ones (19 versus 11%, P= 0.03255). These results are shown in TABLE I.

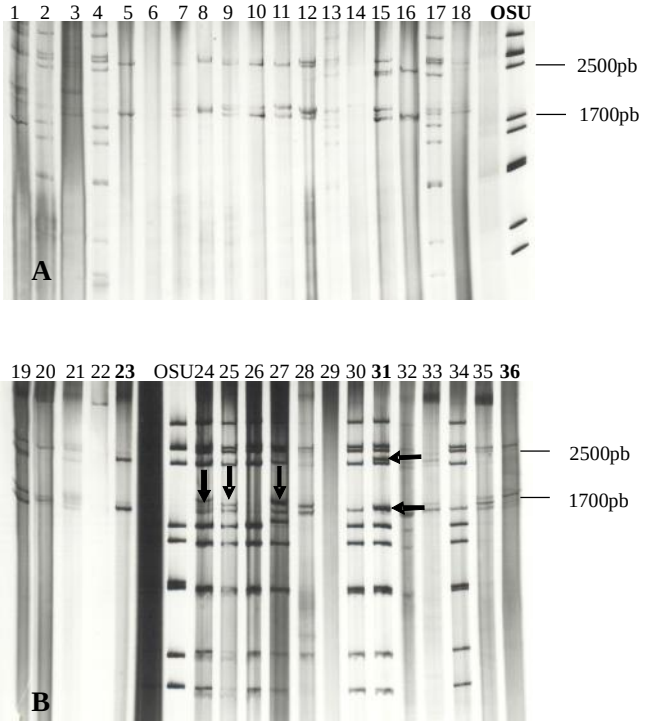

FIGURE 1. ELECTROPHORETIC PATTERNS OBSERVED IN DIFFERENT PBV ISOLATES FROM PIGLET STOOL SAMPLES. ELECTROPHORESIS ON 7% POLYACRYLAMIDE GEL OF DSARN EXTRACTED FROM 1 TO 2 WEEKS OLD PIGLET STOOL SAMPLES. A: THERE ARE SLIGHT VARIATIONS IN THE ELECTROPHORETIC BANDS CORRESPONDING TO PBV LANES 5, 8, 16 AND 18: POSITIVE SAMPLES TO PORCINE PBV WITH THE TWO EXPECTED ELECTROPHORETIC BANDS, LANES 7, 9, 10, 11, 12, 15: SAMPLES ARE CONSIDERED DOUBTFUL FOR PBV BECAUSE THE PRESENCE OF DUAL OR MULTIPLE ELECTROPHORETIC BANDS PATTERN, LANES 2, 4 AND 17: SAMPLES POSITIVE FOR ROTAVIRUS. OSU: PORCINE STRAIN OF ROTAVIRUS USED AS A STANDARD. B: ARROWS: MIX EXCRETION OF PBV AND ROTAVIRUS, TWO ADDITIONAL ELECTROPHORETIC BAND ARE OBSERVED BETWEEN GENOMIC SEGMENTS 4 AND 5 OR 3 AND 5 (LANES 24, 25, 27, 31).

To investigate the frequency of PBV excretion in the different age groups sampled, it was calculated the detection frequency of PBV and rotavirus within each age group based on the number of samples taken for each group was calculated, TABLE II shows the detection frequency for PBV and rotavirus

TABLE I
FREQUENCY OF PBV AND ROTAVIRUS FECAL EXCRETION IN PIGLETS

		Positive to		
Condition		PBV*	RV*	PBV+RV
Diarrhea	101 (25%)	7/101 (7%)[a]	19/101 (19%)[c]	2/101 (2%)[e]
Non diarrhea	301 (75%)	43/301 (14%)[b]	32/301 (11%)[d]	8/301 (3%)[f]
Total	402 (100%)	50/402 (12%)	51/402 (13%)	10/402 (2%)

*Detection of viral agents was made by PAGE on the basis of characteristic electrophoretic bands. PBV: Picobirnavirus; RV: Rotavirus. a, b: P = 0.0526065, c, d: P = 0.0325573, e, f: P = 0.7051709.

TABLE II
FREQUENCY OF PBV AND ROTAVIRUS EXCRETION IN RELATION TO AGE

Age group (days)	PBV[a] n (%)	Rotavirus[a] n (%)
7 - 14	17/59 (29%)	6/59 (10%)
15 - 28	25/201 (12%)	41/201(20%)
29 - 42	1/73 (1%)	3/73 (4%)
43 - 56	7/69 (10%)	1/69(1%)

[a] Detection of viral agents was made by PAGE on the basis of characteristic electrophoretic bands. PBV: Picobirnavirus.

by age group. PBV as well as rotavirus excretion was observed for all age groups. However, the frequency of virus excretion dropped abruptly for both viral agents after d 29 of age (P <0.05). For PBV, the excretion frequency was higher in samples from animals between 7 and 14 d old (29%), while for rotaviruses the higher detection frequency was observed in the group between 15 and 28 d old (20%).

The frequency of PBV detection obtained in this work (12%) was very similar to those previously reported by Ludert *et al.* [25], 10%, and Gatti *et al.* [17], 11% for samples collected also from piglets. Furthermore, it is very similar to the detection frequency obtained in this investigation for rotavirus (13%). The observed frequencies of fecal excretion suggest endemic levels for PBV within piglet populations. However, in contrast to rotavirus, and in agreement with a previous work [25], no significant differences were found in the detection frequency of PBV in samples collected from animals with or without diarrhea. Such evidence suggests that PBV is not a cause of diarrhoea in piglets. However, these results contrast with those reported by Gatti *et al.* [17] whom found significant association between diarrhoea and excretion of PBV. The diarrhoea suffered by piglets can be of differents etiologies and can respond to multifactor causes. Diet, post-weaning stress, viral and bacterial infections, and parasitic infestations are well known causes of diarrhoea in piglets. These factors vary from farm to farm and could partially explain the disease association differences between this work and that of Gatti *et al.* [17]. Another possible explanation is that PBV behave in pigs as an opportunistic agent that cause diarrhoea only under certain circumstances, as have been suggest for HIV infected humans [18, 20, 22].

The detection of PBV in fecal samples from animals with and without diarrhoea in equal frequencies, strongly suggests that PBV is not a causative agent of diarrhoea in animals. However, the available information so far does not offer conclusive evidence for the PBV pathogenicity and additional research in this regard is still needed. Also, it is very interesting the detection of PBV in respiratory tract from humans and pigs, suggesting that picobirnaviruses might be not only potential pathogens but also respiratory pathogens [37, 38]. The wide host range and dissemination of PBV pose an interesting study challenge, especially because very little is known about their biology. As stated by Wang *et al.* [41], “the extreme stability and resistance to treatment of PBVs make these viruses ubiquitous. Thus, a waterborne route of PBV acquisition could explain how the closely related strains are acquired by different host species in the same setting”. The characteristics of the viral particle, with two small genomic segments, encoding for only the viral polymerase and the capsid protein known so far, suggest that PBV is a highly efficient virus in terms of genomic replication and adaptation.

Electron microscopy of purified virus particles

In order to visualize by electron microscopy (EM) PBV viral particles present in positive samples, viral concentrated suspensions were prepared and purified from samples that resulted strongly positive to PBV by PAGE. The FIG. 2 shows electron micrographs of PBV purified virus particles, which constitudes one of the very few electrone micrographs published until now. In these preparations, enrichment (more than 15 viral particles per field) in viral particles whose ultrastructure was compatible with PBV was observed. Particles were spherical, with approximately 35 nm in diameter, non-enveloped, and with uniform structure and morphology, suggestive of icosahedral symmetry. The unique architecture of PBV have been established. The virions display a triaconta hedral desing, where the coat protein dimers form a rhombus concave in shape giving raise to a spherical particle with this particular architecture [12]. In addition, structures suggestive of empty viral cupids and, given the nature of the samples, the presence of viral particles similar to bacteriophages, as well as

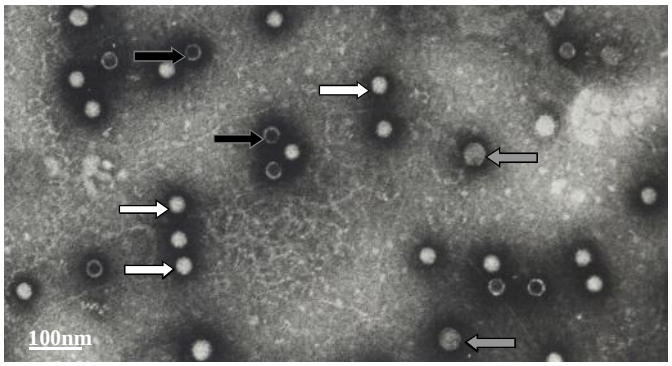

FIGURE 2. ELECTRON MICROGRAPH OF CsCl PURIFIED PBV VIRUS PARTICLES. PARTICLES WERE NEGATIVELY STRAINED WITH 2% PHOSPHOTUNGSTIC ACID. WHITE ARROWS: COMPLETE PBV VIRAL PARTICLES. BLACK ARROWS: EMPTY PBV CAPSIDS. GREY ARROWS: LARGE VIRUS PARTICLES, PRESUMABLY BACTERIOPHAGES. MAGNIFICATION 19000X.

impurities of a nature different to viruses, were observed. On note, the preparations observed by EM were subjected to PAGE analysis after DNase I and RNase T1 treatments and were found to be highly enriched in PBV genomic dsRNA (data not shown).

PBV electrophoretic variability

The analysis by PAGE of the genome of different PBV isolates revealed variations in the migration patterns for genomic segments 1 and 2. Nearly in all cases segment 1 migrated between OSU rotavirus segments 3 and 4, while segment 2 between OSU rotavirus segments 4 and 5. The observed migrations patterns correspond to estimated sizes for the genomic segment 1 and 2 of 2.5 and 1.7 kbp, respectively (FIG. 1). The presence of double or multiple bands at the corresponding position for each genomic segment was also observed, suggesting the coinfection of a single animal with different PBV strains (FIG. 1A). Analysis by RT-PCR indicated that these samples were indeed positive for PBV (data not shown). More recently, Ganesh *et al.* [16] detected mixed infection of human picobirnavirus when PBV genome analysis by PAGE showed the presence of multiple electrophoretic bands with slight variation in migration pattern. Finally, some samples showed “narrow” electrophoretic patterns where both bands were located between OSU rotavirus segments 4 and 5, ranging in size between 1.7 and 1.9 kbp for genomic segment 1 and 2, respectively (FIG. 1B). Belongs to non diarrhoeic piglets, sugesting no association between.

One of the most notable features of PBV is the heterogeneity observed in the genome electrophoretic pattern. It has been reported by Gatti *et al.* [17], Ludert *et al.* [25] and Gallimore *et al.* [14,15], for both PBV genomic segments isolated from stool samples of pig, rabbit and human. Gallimore *et al.* [15], established three groups of genomic profiles for PBV isolated from rabbits, and detected differences between serotypes by solid phase immune electron microscopy. These results were taken as evidence for the existence of different strains of PBV and to suggest that PBV was a highly variable virus. Molecular epidemiology studies have shown also a large variability at the nucleotide sequence level of PBV human isolates, which were classified into two genogroups [2, 28, 36], a finding that further confirms the existence of different strains or variants of PBV circulating among different mammalian species. However, the relationship between electrophoretic patterns and genetic groups is unknown. Despite genetic diversity, high sequence identities among simian PBVs and between simian PBVs and other genogroup (human and porcine PBV strains) suggest that PBV, is readily transmitted intra and interspecies [41, 42].

A drawback in detecting PBV by PAGE is the observation of electrophoretic bands with very narrow electrophoretic pattern that casts doubts when deciding qualitatively the presence of a PBV genome. Narrow patterns or small genome profile have been observed in human PBV isolates. Indeed, narrow patterns in human have been found associated with diarrhoea; moreover, small genome profile have been detected as the only pathogen among acute watery diarrhoea cases in childrens from one month to six years of age [4, 14]. The data of this work indicate that narrow patterns also could occur for porcine PBV.

CONCLUSIONS

These results confirm that PBV circulates frequently in piglets, but their pathogenic potential remains elusive. The slight electrophoretic variation of PBV genome segments suggest close genetic relationships between PBV's circulating in Venezuelan farms. Aditionally, narrow patterns also could occur for porcine PBV but no evidence for pathogenicity was observed. Regardless to the observed frequencies of fecal excretion, these results suggests endemic levels for PBV circulation within piglet populations.

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