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Identification of human Norovirus (HNoV) in domestic pig stool samples

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Abstract

Objective. To determine the presence of NoVs as a possible causal zoonotic agent of acute diarrhea in pigs and humans. **Materials and methods.** We collected a total of 77 samples from diarrheal children under 5 years and pigs under 2 months from La Chamba town in Tolima, Colombia. These samples were transported to the Laboratory of Virology of the Pontificia Universidad Javeriana in Bogotá, and extraction with Trizol-reagent was done following the manufacturer's instructions. After obtaining the RNA, the next step was to perform RT-PCR for obtaining the expected amplification product of 213- bp NoVs. Finally, the positive samples obtained in the RT-PCR were sequenced and analyzed by bioinformatics methods. **Results.** Six positive diarrheic samples from children and a positive diarrheic sample from pigs were detected by a band of 231 bp. Five of the six positive samples in children and the positive pig sample were sequenced and analyzed. **Conclusion.** Given the close genetic relationship between pig and human sequences, this could be an indication of the potential existence of a common animal acting as a reservoir for human or other animal strains.

Key words: Human Norovirus, domestic pigs, diarrhea, zoonotic transmission, reservoirs

Resumen

Identificación de Norovirus Humano (HNoV) en muestras de estiércol de cerdos domésticos. Objetivo: determinar la presencia de NoVs como posible agente zoonótico causal de diarrea aguda entre cerdos y humanos. **Materiales y métodos:** se recolectaron un total de 77 muestras diarreicas provenientes de niños menores de cinco años y de cerdos menores de dos meses de la población La Chamba en el Tolima, Colombia. Estas muestras fueron transportadas al Laboratorio de Virología de la Pontificia Universidad Javeriana en Bogotá, donde inicialmente se les realizó extracción con Trizol-reagent, siguiendo las instrucciones del fabricante. Una vez obtenido el RNA, el siguiente paso fue hacer la RT-PCR para obtener el producto de amplificacion esperado para NoVs de 213 pb. Finalmente, las muestras positivas obtenidas en la RT-PCR fueron secuenciadas y analizadas mediante métodos bioinformáticos. **Resultados:** se obtuvieron seis muestras positivas de diarrea de niños y una muestra positiva de diarrea de cerdos, las cuales se evidenciaron en una banda de 231 pb. Cinco de las seis muestras positivas en niños y la muestra positiva en cerdos fueron secuenciadas y analizadas. **Conclusiones:** dada la estrecha relación genética que se evidencia entre las secuencias del cerdo y el humano, este podría ser un indicio de que exista la posibilidad de un animal en común como reservorio para cepas de humano u otras cepas de animales.

Palabras clave: Norovirus humano, cerdos domésticos, diarrea, transmisión zoonótica, reservorios

Resumo

Identificação de Norovírus Humano (HNoV) em amostras de suínos domésticos. Objetivo. Determinar a presença de NoVs como possível agente zoonótico causal de diarréia aguda entre porcos e seres humanos. **Materiais e métodos.** Foram coletadas um total de 77 amostras de crianças diarréicas menores de cinco anos e porcos com menos de dois meses da população "La Chamba" Tolima-Colômbia. Estas amostras foram transportadas ao laboratório de virologia da Pontifícia Universidade Javeriana - Bogotá, onde foram inicialmente submetidas à extração com Trizol reagment e seguindo as instruções do fabricante, após a obtenção do RNA o próximo passo foi realizar a RT-PCR para obter o produto de amplificação esperado para NoVs de 213 bp. Finalmente as amostras positivas obtidas no RT-PCR foram seqüenciadas e analisadas por métodos de bioinformática. **Resultados.** Foram obtidas seis amostras positivas de diarréia nas crianças e uma amostra positiva de diarréia em suínos, as que foram representadas em uma banda de 231 pb. Cinco das seis amostras positivas em crianças e a amostra positiva em suínos foram seqüenciadas e analisadas. **Conclusões.** Dada a estreita relação genética que se manifesta entre as seqüências de suínos e humanos, isso poderia ser uma indicação de que existe a possibilidade de um animal comum como reservatório para o humano ou outras cepas de animais.

Palavras-chave. Norovírus humano, porcos, diarréia, transmissão zoonótica, reservatórios.

Introduction

NoVs are recognized as common agents responsible for diarrhea around the world affecting children, adults and the elderly. Although the prevalence of NoV-associated gastroenteritis is well defined in developed nations (1), little is known regarding the incidence of NoV-associated disease in developing countries. Some studies have been carried out in different regions in Colombia claiming that NoVs are responsible of 10% of acute gastroenteritis in children (2-3). The high prevalence of NoV has been attributed to its low infectious dose, environmental resistance, strain diversity, asymptomatic and prolonged shedding, and diverse transmission vehicles (1, 4).

Family Caliviridae includes non-enveloped viruses with single-stranded positive sense RNA classified into 4 genera by the International Committee on the Taxonomy of Viruses (ICTV) in 2002: Norovirus, Sapovirus, Vesivirus and Lagovirus (5). Recently, a fifth genus that affects bovines has been proposed and provisionally named Nabovirus or Becovirus (6). NoVs, Sapoviruses and the unclassified bovine viruses cause gastroenteritis in humans and other animals. Vesiviruses can cause disease in humans and swine while Lagoviruses cause diseases in rabbits.

NoVs are further classified into five genogroups (GI to GV) based on the sequence of the viral capsid protein VP1, of which GI, GII and GIV affect humans (7). GII viruses, the predominant genogroup causing gastroenteritis worldwide, can infect human and swine, while GIII are found in bovines and GV viruses have murine tropism (8). Furthermore, GII viruses have shown to be genetic and antigenically related to swine strains, raising the possibility of zoonotic behavior and suggesting swine livestock as a viral reservoir that could contribute to the spread of the virus into human populations (4, 5, 9). Diarrhea by NoVs has shown a closer relationship with communities that share common places where they are in close contact with each other, for instance hospital and military wards, schools and cruise ships (5).

The purpose of this study was to detect NoVs in diarrheic samples from piglets and children and to analyze their phylogenetic relationships to support a possible zoonotic transmission between species.

Materials and methods

Diarrheic samples were collected from 77 children (coded as NN) under 5 years and 39 piglets (coded as NNC) under 2 months old from villages close to a small town known as La Chamba in Tolima, Colombia. Samples were taken from the ground immediately after pigs finished their depositions. The main economic activity of the population in this region is pottery; additionally, some inhabitants breed pigs in their own homes as an economic alternative for an extra income, a practice they denominate as "pig backyard breeding".

Total RNA was extracted from diarrheic samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, RT-PCR was carried out using the protocol described by Trujillo et al., (10) which includes a set of degenerate primers (MON 431, 432, 433 and 434) annealing to the 3' end of the ORF1 that corresponds to RNA-polymerase gene. PCR products were separated by electrophoresis and visualized under a UV lamp after ethidium bromide staining. In total, six human and one pig samples were PCR-positive (213bp). The same samples were used to find Astrovirus.

PCR products were then sequenced by Macrogen, Inc. Six sequences were obtained (GenBank accession numbers: GU474950, GU474951, GU474952, GU474953, GU474954 and GU474955), five from humans (NN1, NN13, NN14, NN34, NN35) and one sequence from pig (NC9). Then, they were entered to the Mega 4 program (www.megasoftware.net) together with 31 prototype sequences of NoVs to build a dendrogram using the p-distances method and the Kimura-2-parameter with a bootstrap of 1000.

Results

The resulting phylogenetic tree showed that all sequences analyzed correspond to human NoVs (bootstrap 100) and that they are closely related to each other (Figure 1); however, the cluster formed by these strains is anchored in a different cluster to the other human and porcine prototype NoVs included. Moreover, the strain detected in the piglet (NC9) is closely related to two of the strains detected in children (NN1 and NN34), as was demonstrated by the high bootstrap values observed in the tree (97 and 100). Some samples were positive for Astrovirus. These findings are published elsewere (11).

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units

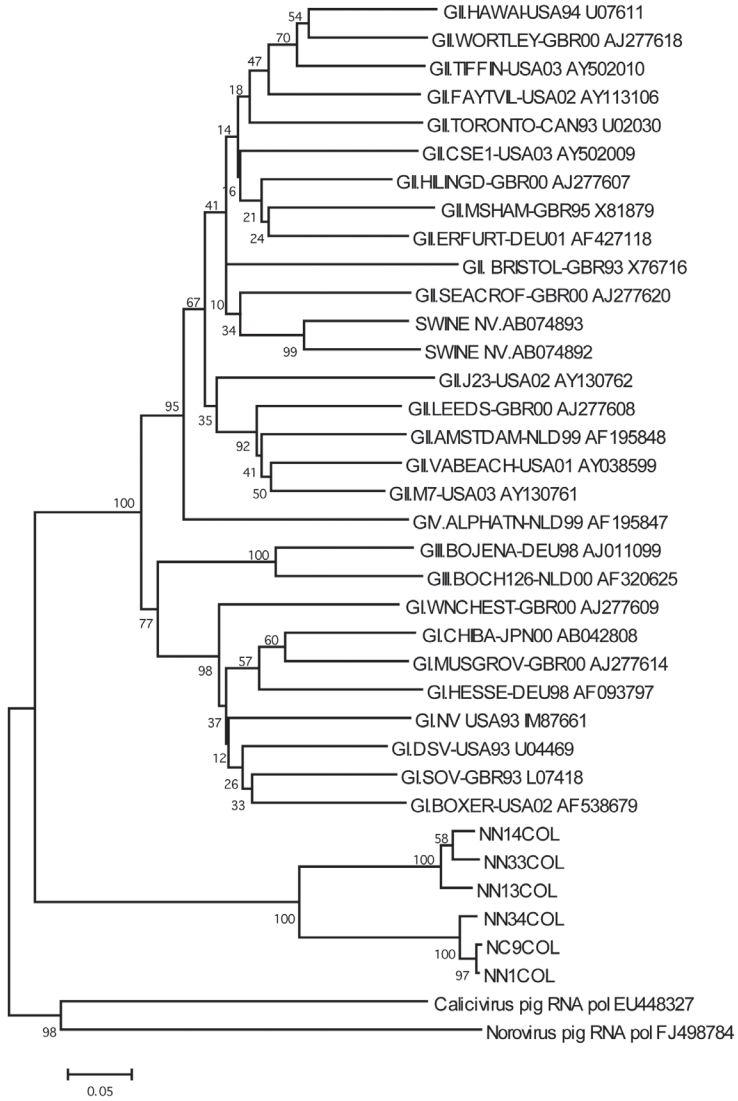

Figure 1. Phylogenetic tree built with 30 prototype strains reported at Genbank. The NC9 was obtained from a piglet stool sample but here it appears in a cluster with NN3, which belongs to a child.

as those of the evolutionary distances used to infer the phylogenetic tree. All the positions containing gaps and missing data were eliminated from the dataset (complete deletion option) having as a result a total of 194 positions in the final dataset. Phylogenetic analyses were done with MEGA4 (12).

Discussion

La Chamba is a low-income town located in central Colombia. In this town, pigs are treated as pets and have no space restrictions; for instance, children often play with them and conduct their daily lives with them around, meaning that these environmental conditions can facilitate the transmission of pathogens from pigs to humans and thus increasing the risk of zoonotic disease transmission.

The presence of human NoV strains in pig stool samples can be explained by the existence of possible emerging variants of NoVs that could infect and replicate in pigs. Previous reports suggest that subclinical infections of human NoVs are seen in pigs and viruses can persist in this host, thus increasing the risk of recombination with swine strains (4, 13). Moreover and although GII.4 strains can infect piglets experimentally (14), these data indicate that such infections may occur naturally as well.

The close genetic relatedness of porcine and human sequences within genogroup II viruses indicates the possibility of a common animal reservoir for human or other animal strains (4). Genetically related viruses are commonly found in different species without resulting in apparent widespread interspecies transmission. A publication of Di Martino *et al*. showed the presence of a feline Calicivirus in dogs with gastroenteritis; however the epidemic impact of diarrhea caused to other hosts different than feline is unknown.

The same phenomenon was observed in sheep and cows where NoV-GIII strains remained in those animals, becoming reservoirs for the virus and contributed to the transmission of viruses among animals (15). Additional epidemiological studies must be carried out in swine to monitor the antigenic and genetic variations among these viruses for a better assessment of their prevalence and potential zoonotic transmission.

Conclusion

The situation described in this study is particular to developing countries, where the instauration of better hygienic practices can have a great impact by decreasing the burden of infectious diseases such as infectious diarrhea. We hope that our results will raise awareness on the need of better hygienic practices among humans that co-exist with animals, and will set the ground for further studies aimed to determine NoV zoonotic transmission.

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Conflict of interest

The authors declare no conflict of interest regarding this study.

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