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A Novel Picornavirus Associated with Gastroenteritis▽†

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A novel picornavirus genome was sequenced, showing 42.6%, 35.2%, and 44.6% of deduced amino acid identities corresponding to the P1, P2, and P3 regions, respectively, of the Aichi virus. Divergent strains of this new virus, which we named salivirus, were detected in 18 stool samples from Nigeria, Tunisia, Nepal, and the United States. A statistical association was seen between virus shedding and unexplained cases of gastroenteritis in Nepal (*P* = 0.0056). Viruses with approximately 90% nucleotide similarity, named klassevirus, were also recently reported in three cases of unexplained diarrhea from the United States and Australia and in sewage from Spain, reflecting a global distribution and supporting a pathogenic role for this new group of picornaviruses.

The falling cost of DNA sequencing has led to a recent surge in human and animal virus discoveries (1–3, 5–12, 14, 16–17, 19–24, 27, 30, 31, 33, 39, 43, 44). While the pathogenicity of some newly characterized human viruses has been demonstrated, it remains unknown or controversial for other viruses, which may be commensal or pathogenic in only a very small fraction of infections (25, 32, 40, 42, 45). Genetic characterization of previously unknown viruses allows the rapid design of nucleic acid tests needed to determine their association with different medical conditions, their presence in different populations, and the design of antibody tests for determining seroprevalence (25, 28, 34, 35, 47).

Using sequence-independent PCR amplification, pyrosequencing, and sequence similarity searches (46) (see the text in the supplemental material), we analyzed the virus sequences present in 95 stool samples from Nigerian children suffering from nonpolio acute flaccid paralysis (AFP). Sequences derived from a 10-month-old female child exhibiting right-side asymmetric sudden flaccid paralysis (patient no. NG-J1) formed a 6,981-bp contig consisting of 2,903 individual sequence reads, which was distantly related to sequence of the *Aichi virus* species in the *Kobuvirus* genus of the *Picornaviridae* family (48, 49). Similar sequences were also observed in a second, 24-month-old patient with right-side asymmetric sudden flaccid paralysis (patient no. NG-F1). Gaps between sequenced viral fragments were connected by nested reverse transcription-PCR (RT-PCR), while the 5′ and 3′ extremity sequences were acquired using primers designed over conserved regions of bovine, porcine, and human kobuviruses. We temporarily named these viruses saliviruses (*s*tool *A*ichi-*li*ke *v*iruses).

The resulting salivirus genome, NG-J1, was 7,124 bp in length with a GC content of 57%, excluding a poly(A) tail. NG-J1 contained a large open reading frame of 7,125 bp encoding a putative polyprotein precursor of 2,374 amino acids (aa), a 5′ untranslated region (UTR) of 709 bp, and a 3′UTR of 148 bp (Fig. 1).

NG-J1 and NG-F1 were highly similar, with nucleotide similarities of 94% and 95% in the P1 and P3 regions, respectively. Salivirus NG-J1 had approximately 90% nucleotide similarity to the recently described klasseviruses (14, 16). Comparison of NG-J1 with the kobuviruses and other picornaviruses showed it was most closely related to Aichi virus, with average amino acid similarities of 42.6%, 35.2%, and 44.6% for the P1, P2, and P3 regions, respectively (see Table S1 in the supplemental material). According to the International committee on Taxonomy of Viruses (http://www.picornastudygroup.com/definitions/genus_definition.htm), the members of a picornavirus genus should share >40%, >40%, and >50% amino acid similarities in their P1, P2, and P3 regions, respectively. Salivirus is therefore at the borderline between a new genus and a highly divergent species in the kobuvirus family. Phylogenetic analysis based on P1 and 3D using the neighbor-joining method with the amino acid *p*-distance model and 1,000 bootstrap replications also showed that NG-J1 and F1 were most closely related to kobuviruses as a deep-rooted lineage (Fig. 2; see also Fig. S1 in the supplemental material). Phylogenetic trees built using other methods produced similar results (data not shown).

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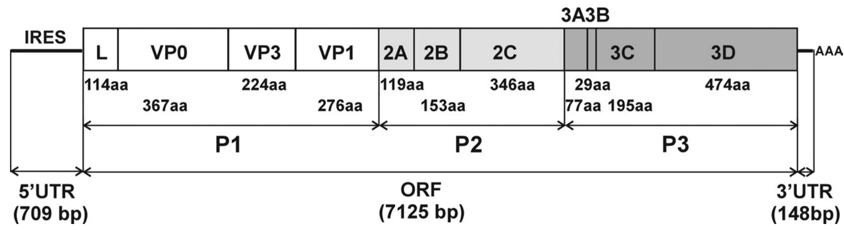

FIG. 1. Genome organization of human salivirus NG-J1.

The length of the 5′ UTR for salivirus (at least 709 nucleotides) was comparable to those of Aichi virus (712 to 744 bp), porcine kobuvirus (576 bp), and bovine kobuvirus (808 bp). The viral GC content of 57% was close to those of kobuviruses and aphthoviruses but higher than those of enteroviruses, rhinoviruses, hepatoviruses, and cardioviruses. The region containing the 5′ UTR internal ribosome entry site (IRES) (~420 bp) shared 71% similarity with that of Aichi virus, and the

FIG. 2. Phylogenetic relationship of saliviruses with representative species from each picornavirus genus, based on amino acid similarity of P1 region using neighbor-joining method with *p*-distance and 1,000 bootstrap replications.

TABLE 1. Frequency of salivirus identified by RT-PCR analysis of stool samples

Case description	No. positive/no. tested (%)	
	Diseased	Healthy control/contact
Nigerian AFP	5/93 (5.4)	NA[a]
Tunisian AFP	3/96 (3.1)	0/96
Minnesotan gastroenteritis	2/96 (2.1)	0/96
Nepali gastroenteritis	8/92 (8.7)	0/96

[a] NA, not available.

location of the pyrimidine tract (691 to 702 bp) was very close to the initiation methionine (710 to 712 bp), suggesting that the salivirus IRES belongs to the type II IRES group, as do those of kobuviruses, aphthoviruses, hepatoviruses, and cardioviruses (15, 48). The extreme 5′ end of the 5′ UTR (~250 bp) had no significant match in GenBank using a Blastn search. The secondary structure of the 5′ UTR of salivirus, predicted by the Mfold software program (53), was highly structured, and hairpin structures were found at the extreme 5′ end, as with Aichi virus.

Based on comparisons with the Aichi virus and other kobuviruses, the polyprotein of salivirus NG-J1 comprised a putative leader protein (L), capsid proteins VP0, VP3, and VP1, and nonstructural proteins 2A to 2C and 3A to 3D (Fig. 1). The putative cleavage sites are L/VP0 (Q/G), VP0/VP3 (P/Q), VP3/VP1 (Q/S), VP1/2A (Y/S), 2A/2B (Q/G), 2B/2C (Q/G), 2C/3A (Q/G), 3A/3B (Q/G), 3B/3C (Q/G), and 3C/3D (Q/S). The L protein (114 aa) was shorter than those of kobuviruses (170 to 195 aa) and had no significant similarity with that of kobuviruses. A notable feature was the 2A protein, which did not contain the HBox/NC and hydrophobic domains thought to be involved in control of cell growth and conserved in Aichi virus and porcine and bovine kobuviruses, parechoviruses, and avian encephalomyelitis virus (in the hepatovirus genus) (18). No significant similarity was seen between the salivirus 2A protein and any other protein in GenBank. Based on the detection of H, C, and D catalytic triad residues at approximately the same 2A location as in rhinoviruses and enteroviruses, we postulate that the salivirus 2A protein is a trypsin-like protease (18). A putative VPg was identified with a YSG tyrosine-containing motif starting at the third amino acid position, providing the Y covalently bound to the 5′ end of picornavirus genomic RNA. At VPg amino acid position 21, another YSG motif was seen, immediately preceded by Q/PV, possibly reflecting the presence of a protease cleavage site at Q/P, followed by another third-position Y, potentially resulting in a second, shorter, 11-amino-acid-long VPg. The NPG/P motif seen in some picornavirus genera between the 2A and 2B proteins, which induces 2A release from the extending polyprotein chain on ribosomes, was not detected in salivirus. The integrin binding RGD motif was not detected in VP1. The 3D RNA-dependent RNA polymerase region contained the highly conserved KDELR, YGDD, and FLKR amino acid motifs.

Prevalence studies were carried out using RT-nested PCR to understand the geographic distribution and possible association of saliviruses with either nonpolio AFP or gastroenteritis (see the text in the supplemental material for cohort descriptions). A nested set of PCR primers were used targeting a 3D region (SAL-F1, 5′-GAAGATGCCATTCGTGGTCTC; SAL-R1, 5′-AGTCCAGAACACGACCAGGTT; SAL-F2, 5′-CTTTCCCAATCTCCTGGCTAC; and SAL-R2, 5′-GAAGGACAGAGGGGATAGTGG) (see the text in the supplemental material for PCR conditions). Saliviruses were detected and sequenced partially in 18 samples from four countries (Nigeria, Tunisia, Nepal, and the United States) (Table 1 and Fig. 3). Amino acid differences between saliviruses in the conserved 3D region targeted for the diagnostic PCR ranged up to 12%,

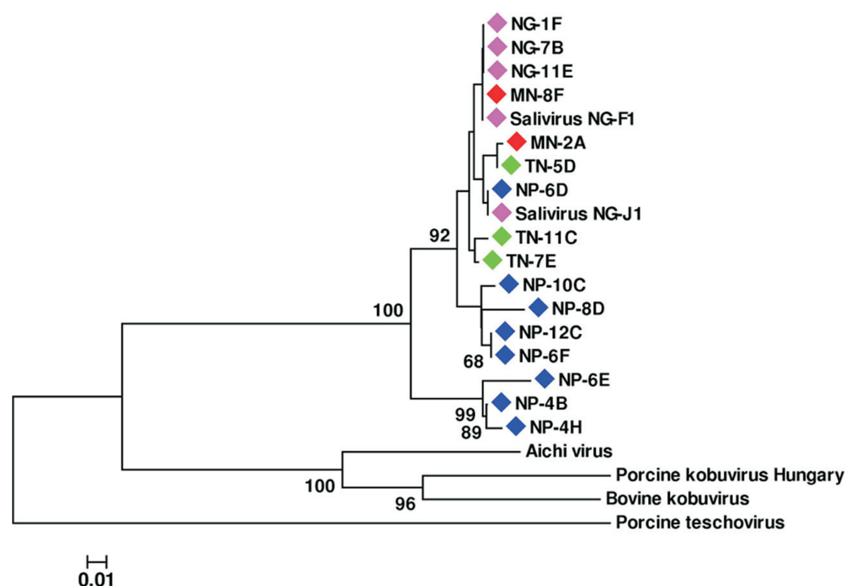

FIG. 3. Phylogenetic analysis of saliviruses from four sample cohorts based on the translated partial 3D region amplified by diagnostic nested RT-PCR. NG, Nigeria, TN, Tunisia, NP, Nepal, MN, Minnesota.

indicating considerable genetic diversity among saliviruses. Saliviruses were found in the stool of 5/93 (5.4%) children with nonpolio AFP from Nigeria and in 3/96 (3.1%) cases from Tunisia. Saliviruses were not detected in the stool samples from healthy Tunisian children who had been in close contact with nonpolio AFP cases (i.e., contacts). The difference in prevalence between the nonpolio AFP and healthy contact groups from Tunisia did not reach statistical significance. No control group was available from Nigeria. The highest prevalence of saliviruses was seen in 8/92 cases of unexplained gastroenteritis from Nepal (8.6%), while no positives were found in 96 healthy matched controls from Nepal (Table 1). A statistically significant association was measured ($P = 0.0056$) using Fisher's exact test (two-tailed) between salivirus detection and gastroenteritis in Nepal. Of the 96 stool samples from Minnesotan unexplained gastroenteritis, 2 were positive for salivirus, while none of 96 healthy controls were shedding saliviruses. The limited number of cases and small group sizes preclude a definitive conclusion on salivirus' association with diarrhea in the U.S. cohorts. The detection of saliviruses in nonpolio AFP cases, as in a healthy U.S. child (14), indicates that not all salivirus infections are associated with diarrhea.

Aichi viruses have been associated with gastroenteritis, often in mixed infections with other enteric viruses, and a high seroprevalence has been reported (4, 13, 29, 36, 41, 48–52). Kobuviruses have also been detected worldwide in both pigs and cows and were also associated with diarrhea (26, 37, 38). The detection of saliviruses in Nigeria, Tunisia, Nepal, and the United States reported here and recently in the United States, Australia, and Spain (14, 16) shows that these viruses are very widely spread (16). The large degree of genetic diversity among some saliviruses based on partial 3D sequencing may reflect the presence of multiple species and genotypes that may exhibit different biological properties and antibody neutralization profiles. The association between salivirus shedding and diarrhea reported here indicates that such infections may account for a significant fraction of the unexplained cases of diarrhea occurring worldwide.

Nucleotide sequence accession numbers. Sequences determined in this work have been submitted to GenBank under the following accession numbers: for NG-J1, GQ179640; for NG-F1, GQ507022; and for saliviruses in 18 samples from Nigeria, Tunisia, Nepal, and the United States, GQ507006 to GQ507021.

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