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Longitudinal Study of Molecular Epidemiology of Small Round-Structured Viruses in a Pediatric Population

PAUL N. LEVETT,[1]† MARIE GU,[1] BO LUAN,[1] MARGARET FEARON,[2] JOAN STUBBERFIELD,[2] FRANCES JAMIESON,[1] AND MARTIN PETRIC[1]*

Virology Laboratory, Department of Microbiology, Hospital for Sick Children,[1] and Laboratory Services Branch, Ontario Ministry of Health,[2] Toronto, Ontario, Canada

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Small round-structured viruses (SRSV), recently designated members of the family *Caliciviridae*, can now be readily subtyped by amplification of a defined portion of their genome by reverse transcription-PCR and then by identification of the amplicons with specific probes by Southern blotting. A longitudinal survey (from 1991 to 1995) was conducted to determine the genotypes of the SRSV present in pediatric stool specimens from patients with sporadic cases of gastroenteritis. It was found that almost all viruses were of the G-2 genotype, and on probing, the subtype P2-B was predominant but the frequencies of the different subtypes varied from year to year. A survey of the genotypes of SRSV from community outbreaks from 1995 showed that the G-2 genotype was also predominant and that the distribution of its subtypes was similar to that seen in sporadic cases of diarrhea in pediatric patients over the same time period. It was concluded that there is a succession of subtypes of SRSV in our pediatric population over time. This distribution of genotypes in sporadic cases of pediatric gastroenteritis may reflect the distribution in community outbreaks occurring at the same time.

Small round-structured viruses (SRSV) are well-established agents of gastroenteritis that were first described with the identification of Norwalk virus in 1972 (11; for a review, see references 9 and 10). These were initially referred to as Norwalk viruses and Norwalk-like viruses and included agents designated as Montgomery County, Hawaii, Snow Mountain, and Taunton (5, 9, 10, 11). On the basis of the sequence analysis of the Norwalk and Southampton virus genomes and their antigenic and structural features, these agents have been recently reclassified into the family *Caliciviridae* (4, 7, 8, 13, 14). Four distinct groups of SRSV, documented by solid-phase immune electron microscopy, have been designated UK-1 to UK-4 with the following associations: UK-2, Norwalk virus; UK-1, Taunton agent; UK-3, Hawaii agent; and UK-4, Snow Mountain agent (17, 18). These groups have recently been further subdivided into six antigenic subgroups (19). This grouping has been found to correlate with the nucleic acid sequence of a portion of the viral polymerase gene, which was determined by reverse transcription (RT)-PCR and Southern hybridization (1, 6, 22). This correlation has allowed for the classification of these agents into two genotypes, G-1 and G-2 (1, 24). Subgroups of each genotype can be established by Southern hybridization with specific probes, and these correspond closely with the UK groups (1). Of these, the UK-2 group is placed into G-1, whereas most of the other groups are placed into G-2.

Viruses whose morphologies were consistent with that of SRSV have been detected at the Hospital for Sick Children, Toronto, Canada, since they were first reported in 1977 as "minireoviruses" (20). The genome of one such virus has been partially sequenced, and its genomic organization was shown to be consistent with that of the *Caliciviridae* (16). In this study, the RT-PCR assay and probing described by Ando et al. (1) were applied in a longitudinal survey to determine the degree of genomic diversity in the SRSV diagnosed from the patient population in our institution from 1991 to 1995 and from a number of community outbreaks in 1995.

MATERIALS AND METHODS

Electron microscopy of fecal specimens. Specimens from patients with symptoms of diarrhea were processed for negative contrast electron microscopy at a magnification of ×50,000 (20). They were examined for the presence of SRSV particles measuring 30 to 40 nm in diameter. The specimens were stored at 4°C for up to 3 weeks. For longer storage, the specimens were divided into aliquots and frozen at −80°C as recommended by Lew et al. (15).

Extraction of RNA from fecal specimens. A 10 to 25% suspension of the stool specimen was prepared in 1 ml of sterile phosphate-buffered saline and clarified by centrifugation at 3,000 rpm in a microcentrifuge for 30 min. The supernatant was recentrifuged for 15 min at 10,000 rpm and further clarified by recentrifugation at 10,000 rpm for an additional 15 minutes.

A 150-μl volume of the clarified stool suspension was added to 1.5 ml of TRIzol reagent (Canadian Life Technologies Inc., Burlington, Canada) in a 2-ml microcentrifuge tube, and the preparation was mixed by repeat pipetting (3). After incubation at room temperature for 5 min, the preparation was mixed by shaking with 0.3 ml of chloroform, and after centrifugation at 12,000 rpm for 10 min, the upper aqueous layer was removed and mixed with 0.75 ml of isopropanol at room temperature for 10 min. The pellet obtained after centrifuging the preparation at 12,000 rpm for 10 min at 4°C was washed with ice-cold 75% ethanol, dried, resuspended in 40 μl of RNase-free water, heated at 60°C for 10 min, and stored at −80°C.

Primers and probes. Primer sets and probes used were the ones described by Ando et al. (1) and designated accordingly. For positive strand synthesis the G-1 set contained primers SR48, SR50, and SR52 and the G-2 set contained primer SR46. Both G-1 and G-2 sets had primer SR33 for negative strand synthesis. The products of amplification with the G-2 primer set were hybridized with probes SR67d (P1-B), SR61d (P2-A), and SR47d (P2-B). Primers and probes were purchased from Dalton Chemical Laboratories Inc. (Toronto, Canada). Probes were labeled with digoxigenin with a digoxigenin oligonucleotide 3′ end labeling kit (Boerhinger Mannheim Canada, Laval, Quebec, Canada).

RT-PCR. RT and PCR were performed as sequential steps in a single 0.5-ml microcentrifuge tube. For the RT reaction, 10 μl of RNA extract was added to 10 μl of the RT mixture to make a solution containing 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM (each) dATP, dCTP, dGTP, and dTTP, 2.6 μM random primer, 20 U of RNase inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase (Canadian Life Technologies Inc.). The reaction mixture was overlaid with sterile mineral oil and incubated at room temperature for 10 min. The RT reaction was performed in a Perkin-Elmer

* Corresponding author. Phone: (416) 813-6111. Fax: (416) 813-5993.

† Present address: University of the West Indies, Queen Elizabeth Hospital, Bridgetown, Barbados.

thermal cycler at 42°C for 30 min and 99°C for 5 min, and then the mixture was soaked at 5°C for 5 min.

The PCR was performed after the addition of 80 μl of the PCR mixture to make a solution containing 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM (each) dATP, dCTP, dGTP, and dTTP, 2.5 U of DNA polymerase (Amplitaq; Perkin-Elmer Cetus and Applied Biosystems Inc.), and 0.15 μM (each) G-1 or G-2 primer. After denaturation at 94°C for 2 min, the reaction mixture was subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min and then incubated at 72°C for 10 min. The reaction mixtures were then held at 4°C until the products were analyzed by electrophoresis in 1.2% agarose gels at 110 V for 45 min. The gels were stained with ethidium bromide and viewed under UV illumination.

Southern hybridization and chemiluminescence detection. After staining with ethidium bromide, the gel was soaked in a denaturing solution of 1.5 M NaCl and 0.5 M NaOH for 20 min with gentle shaking and rinsed with water. The gel was then immersed into a solution of 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl, and blotting was performed by capillary transfer onto positively charged nylon membrane filters (Boehringer Mannheim) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer and exposure to UV light to link the DNA to the membrane. For dot blotting, 1 to 2 μl of the PCR product was applied to a hybond-N membrane filter and, after drying, exposed to UV light.

Hybridization and chemiluminescence detection were performed by protocols recommended by Boerhinger Mannheim Corp. and described by Ando et al. (1). The filters were washed for two 5-min periods with 50 ml of 2× SSC–0.1% sodium dodecyl sulfate (SDS), and the washes were repeated with a solution of 0.1× SSC–0.1% SDS.

For chemiluminescence detection, the membrane filter was washed in buffer 1 (0.1 M malic acid, 0.5 M NaCl [pH 7.5]) and incubated for 30 min in 100 ml of buffer 2 (10% blocking stock solution diluted 1:10 in buffer 1). The filter was then incubated for 30 min in 20 ml of diluted antibody-conjugate solution (diluted antibody conjugate to 150 mU/ml in buffer 2), and the preparation was washed twice for 15 min in buffer 1 and further incubated for 2 min in 20 ml of buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$ [pH 9.5]). Lumi-phos-530 reagent (Boehringer Mannheim) was sprayed on the filter, and after a 15-min incubation at 37°C, the membrane was exposed to a Kodak X-Omat film for various time periods. For rehybridization, the filter was washed in water for 1 min, incubated for two successive 10-min periods in a solution of 0.2 M NaOH and 0.1% SDS at 37°C, and thoroughly rinsed with 2× SSC.

RESULTS

Patients and specimens. Fecal specimens used in the longitudinal study were samples from pediatric patients with gastrointestinal symptoms that had been submitted for diagnostic virology between October 1991 and March 1995 and had diagnosed positive for SRSV by electron microscopy. Of a total of 137 specimens in which SRSV had been detected, 87 specimens from 76 patients were available for this study. The remainder had either dried out during storage or were of insufficient quantity for processing. The average age of these patients was 25.2 months and ranged between 3 and 140 months. Specimens from 52 of these 76 patients had been submitted more than 4 days after admission to the hospital, which indicates that they were likely nosocomial infections. Specimens came from all wards of the hospital, and only four specimens were from outpatients. During this study period, SRSV made up 17.8% of the 768 specimens positive for gastroenteritis viruses, namely, rotavirus, adenovirus, SRSV, and astrovirus.

The data in Fig. 1 show the seasonal prevalence of SRSV, as diagnosed by electron microscopy over the 4-year period. These data demonstrate that the virus may be diagnosed throughout the year, but it is most prevalent in the winter months. On the basis of the monthly incidence of SRSV, the study period was divided into four yearly periods running from September to August.

A second set of specimens positive for SRSV by electron microscopy was obtained after community outbreaks from January to June 1995. These specimens represented a wide range of age groups from pediatric to geriatric, with the majority coming from geriatric facilities.

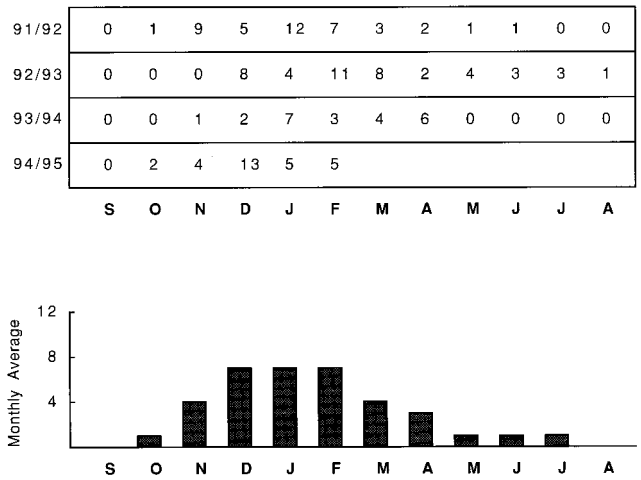

FIG. 1. Monthly incidence of SRSV diagnosed by electron microscopy from 1991 to 1995. Single letters indicate the first letters of the names of the months, beginning with September. Numbers reflect the numbers of cases.

RT-PCR. In the longitudinal survey from 1991 to 1995, with the primer pair designated G-2, a 123-bp amplicon was detected in 70 of 86 specimens that were obtained from 62 of 76 patients. In contrast, with primer pair G-1, an amplicon was detected in only one specimen from these patients. Thirteen specimens from the 76 patients were not reactive in the RT-PCR assay, and of these, 11 had been frozen for more than 1 year. In the community outbreak survey, virus-specific amplicons were obtained from 107 specimens with G-2 primers and 14 amplicons were obtained with G-1 primers.

Shown in Fig. 2 are representative amplification products after agarose gel electrophoresis. These were subjected to Southern blotting and sequential hybridization with probes SR47d (P2-B) and SR61d (P2-A). The probes proved to be specific and were able to identify single subtypes of SRSV. The probes were then used directly on the amplification products in the dot blot format, a subset of which is shown in Fig. 3. The SR47d probe (top panel) hybridized with the majority of the G-2 amplicons, the SR61d probe (middle panel) hybridized with a smaller fraction, and the SR67d probe (bottom panel) hybridized only with the amplicon of a single specimen. This approach enabled us to designate the genotype of SRSV present in each specimen.

Epidemiology of SRSV infection. From the specimens of the 62 patients whose specimens amplified with G-2 primers, the amplicons of 61 patients were successfully blotted and probed. The distribution of G-2 genotypes among these 61 patients is shown in Fig. 4. In each of the years studied a single genotype predominated. In the winter of 1991–1992, genotype P2-B was predominant. In the following winter, all three genotypes were detected, with genotype P2-A being dominant. In the following two seasons, 1993–1994 and 1994–1995, P2-B was again the predominant type with P2-A reappearing at the end of the season. Genotype P1-B was detected in the 1992–1993 and 1993–1994 seasons but as sporadic cases in single patients. Genotype G-1 was detected in a single case in the 1992–1993 season.

In the community outbreaks of the first half of 1995, the G-1 genotype was present in 14 of the 121 specimens analyzed, with G-2 being present in 107, as shown in Fig. 5. Further typing of the G-2 amplicons by the three specific probes demonstrated that the P2-B subtype was predominant; the P2-A subtype was next in prevalence. The P1-B subtype was present in very low

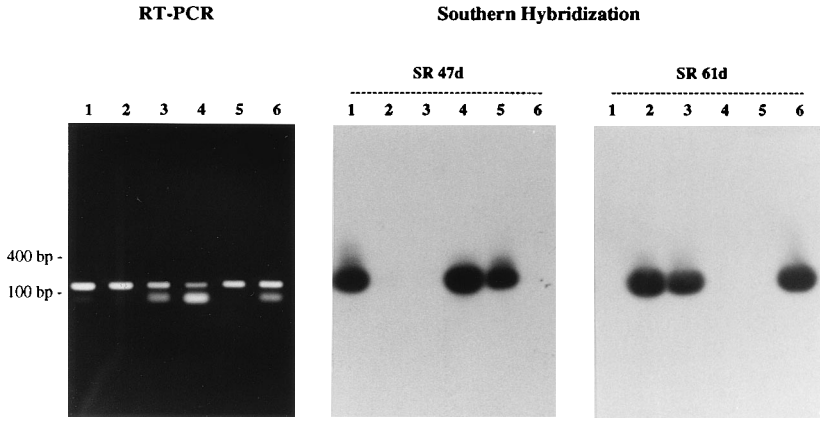

FIG. 2. Ethidium bromide staining and Southern hybridization of RT-PCR products of six representative specimens. Primers for RT-PCR were of the G-2 type.

amounts, with only 3 specimens of 107 being of this subtype. This distribution was strikingly similar to that present in the pediatric population in the 1994–1995 season.

Sequential specimens were available from four immunocompromised infants. In each case the subtype persisted throughout the period of excretion. Four specimens from one infant collected from the 20th to the 28th months of life were all genotype P2-B. This persistence is notable in that the infant had been discharged for a 3-month period between admissions. SRSV was detected in the stool specimens by electron microscopy throughout this period.

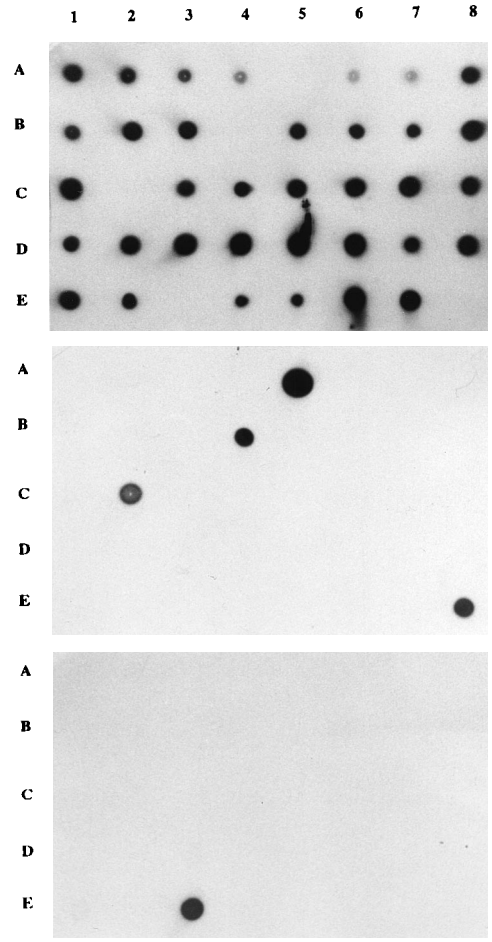

FIG. 3. Probing of RT-PCR products of 40 specimens spotted onto membranes. The top panel was probed with SR47d, the middle panel was probed with SR61d, and the bottom panel was probed with SR67d.

DISCUSSION

The recent developments correlating the genotype of SRSV with specific antigenic types, namely UK-1 to -4, has greatly simplified investigations of the epidemiologic behavior of these agents (1). Although other studies have addressed the diversity of these viruses in outbreaks, our study examined the prevalence of specific genotypes in patients in a single hospital center from a longitudinal perspective and provided an insight into the genotypes present during a portion of this time period in community outbreaks (1, 18, 22, 24).

It has long been known that SRSV infection has a seasonal prevalence (18). Our longitudinal studies in a single pediatric setting have provided evidence that a single genotype of SRSV predominates each winter season. This is in keeping with the seasonal epidemiologic behavior of other viral agents, such as the enteroviruses (21). These observations suggest that, over the winter season, infections with the dominant genotype are

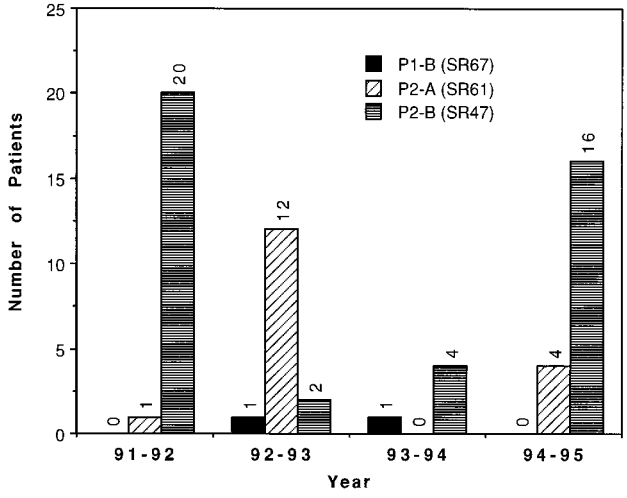

FIG. 4. Annual prevalence of specific G-2 subtypes of SRSV from sporadic cases of gastroenteritis in pediatric patients from 1991 to 1995.

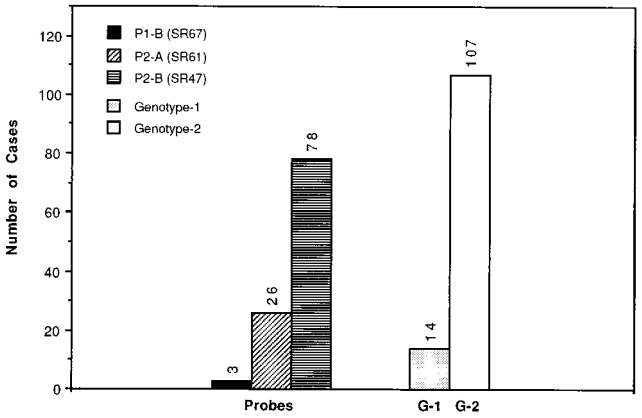

FIG. 5. Prevalence of G-1 and G-2 genotypes and the G-2 subtypes of SRSV from local community outbreaks of gastroenteritis from January 1995 to June 1995.

relatively common and only a subset of patients actually requires medical attention which leads to establishing a definitive virus diagnosis. During this period, it is possible that immunity to the specific genotype develops throughout the population and that the prevalence of infection with that genotype declines and is superseded by a different genotype. This succession of genotypes in fact occurred in our study between the 1991–1992 and 1992–1993 seasons and again in the 1993–1994 to 1994–1995 seasons. On the basis of these observations, one would predict further genotype variations in the forthcoming seasons.

The P2-B genotype proved to be the most common throughout this study. This could be due to the observation that this genotype contains members from a variety of antigenic subtypes, including those from UK-3, UK-4, and some UK-1 (1). A more detailed insight into this may be achieved by expanding the numbers of probes to allow for more type-specific investigations or by sequencing the amplicons (24). Alternatively, the specimens of the genotype could be further examined by the SPEIM assay using reference antisera (17).

The distribution of subtypes of G-2 in community outbreaks for the 1995 season was similar to that present in the pediatric population in the 1994–1995 season. This implies that the subtypes circulating in the population infect the pediatric population as well as all other age groups that are seen in community outbreaks. If in further follow-up studies this hypothesis proves true, then changes in the predominant subtype detected by routinely monitoring the pediatric population could potentially be predictive of the subtypes in community outbreaks.

In immunocompromised patients in our study, SRSV showed evidence of chronic infections. In one patient the virus of the same genotype was shed for an 8-month period. This shedding is in keeping with chronic infections with other gastroenteritis viruses (23).

The method of RNA extraction used in this study differed from that of Ando et al. (1) in that the TRIzol reagent alone was used. This proved adequate for the RT-PCR and showed good concordance with electron microscopic diagnosis. Our approach also differed from that of Ando et al. (1) in that the RT-PCR products were further analyzed by dot blotting rather than by Southern blotting with the appropriate probes. This approach further simplified the system when a larger number of stool specimens were used. Representative specimens were tested by both methods and shown to give equivalent results. Dot blotting allowed for very specific designation of the specimens into the respective genotypes with no evident cross-reactivities, as shown in Fig. 3. The difference in the size of the individual dots most likely reflected the amount of amplicon present for each specimen.

The succession of genotypes of SRSV in a population as shown in this study advances our knowledge of the epidemiologic features of SRSV. Coordinating the genotypes in such settings with community outbreaks promises to provide us with added insight into the behavior of these agents in our population.

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