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Detection and Differentiation of Antigenically Distinct Small Round-Structured Viruses (Norwalk-Like Viruses) by Reverse Transcription-PCR and Southern Hybridization

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Application of reverse transcription (RT)-PCR to detect small round-structured viruses (SRSVs) from fecal specimens of patients with gastroenteritis has been insensitive because of the tremendous sequence heterogeneity between strains. We have designed two RT-PCR primer sets (G-1 and G-2) based on the nucleotide sequence diversity in the RNA polymerase gene of SRSVs belonging to two distinct genogroups represented by Norwalk virus (primers G-1) and Snow Mountain agent (primers G-2). All 22 SRSV strains examined that had been classified previously by solid-phase immune electron microscopy into four antigenic types (UK1, UK2, UK3, and UK4) could be detected by RT-PCR with these two primer sets. The G-1 primer set detected 6 UK2 strains, and the G-2 primers detected 16 strains, including 7 UK1, 5 UK3, and 4 UK4 strains. On the basis of nucleotide sequences of 81-bp fragments of the RT-PCR products from 13 strains determined in this study, together with those previously reported for 17 SRSV strains, we designed four sets of internal oligonucleotide probes (P1-A, P1-B, P2-A, and P2-B) for Southern hybridization, using chemiluminescent detection. The P1-A probe hybridized with PCR products from the UK2 strains; the P1-B probe, with products from two of the seven UK1 strains; the P2-A probe, with four of the remaining five UK1 strains; and the P2-B probe, with products from both UK3 and UK4 strains, as well as with one strain originally typed as UK1 which showed cross-reactivity with UK4 upon retesting by solid-phase immune electron microscopy. RT-PCR with both the G-1 and the G-2 primer sets can increase the detection rate of the many antigenically distinct SRSVs and, when combined with Southern hybridization, may predict the antigenic type of the SRSV associated with infection.

Small round-structured viruses (SRSVs) are single-stranded RNA viruses recently classified in the family *Caliciviridae* (15, 17). The viruses in this group have not yet been cultivated in vitro, and no practical animal model to propagate them has been developed. At least four antigenic types of SRSVs, designated UK1, UK2, UK3, and UK4 (25, 27), have been identified by solid-phase immune electron microscopy (SPIEM), using human clinical samples as the source of antigen and antibody; these antigenic types are similar to the prototype strains Taunton agent (30), Norwalk virus (NV) (19), Hawaii agent (HWA) (34), and Snow Mountain agent (SMA) (6), respectively. These SRSVs are the major cause of food-borne and waterborne outbreaks of nonbacterial acute gastroenteritis (1, 18, 32, 35) and may be a common cause of gastroenteritis in children and adults (18). However, early diagnosis of SRSV infection has been limited by the lack of adequate diagnostic methods. Direct electron microscopy is often not sensitive enough to detect SRSVs, which are usually shed in low concentration (33). Radioimmunoassay (7, 12), enzyme-linked immunosorbent assay (ELISA) (9, 13, 28), and SPIEM (25, 27) to detect SRSVs in stool samples are not routinely performed because the reagents used in these methods are derived from nonreplenishable clinical samples.

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Recently, the entire genomes of NV (15, 17) and Southampton virus (21) were cloned and characterized. The availability of their sequences led many researchers to develop methods to detect SRSVs by reverse transcription (RT)-PCR (4, 16, 29). In initial studies, primers were selected from the RNA polymerase region because of the expected conservation of the sequence among SRSVs of various antigenic types (15, 17). However, examination of SRSVs from numerous outbreaks by RT-PCR, using primers derived from the NV and Southampton virus sequences, resulted in an unexpectedly low detection rate. The low detection rate was suggested to be related to the considerable nucleotide sequence diversity among SRSVs with distinct antigenicity (11, 29). We subsequently confirmed that SRSVs antigenically similar to NV, HWA, and SMA were genetically diverse in the RNA polymerase region and could be classified into two genotypes (genotype 1 for NV and genotype 2 for HWA and SMA) on the basis of this diversity (2). Similar results were obtained by Wang et al. (36).

In this study, we designed two broadly reactive primer sets and four internal oligonucleotide probe sets on the basis of nucleotide sequences within the RNA polymerase region of a variety of SRSV strains. Our goal was to develop an RT-PCR assay to effectively detect these genetically diverse viruses in stool specimens and to differentiate the RT-PCR products according to the antigenic type of the template SRSV by Southern hybridization with chemiluminescent detection.

TABLE 1. SRSV strains used in this study

Strain no.	Identification[a] (code)	Source
1	UK1/1815/91/US (UK1-1)[b]	Elderly, outbreak in nursing home
2	UK1/1079/92/UK (UK1-2)[b]	Child, sporadic case
3	UK1/5605/90/UK (UK1-3)[b]	Child, sporadic case
4	UK1/3723/87/UK (UK1-4)[b]	Child, sporadic case
5	UK1/QEHC11/86/UK (UK1-5)[b]	Child, sporadic case
6	UK1/3453/91/UK (UK1-6)[b]	Elderly, outbreak in geriatric hospital
7	UK1/6708/90/UK (UK1-7)[b]	Child, sporadic case
8	UK2/12121/89/UK (UK2-8)[c]	Adult, sporadic case
9	UK2/8051/92/UK (UK2-9)[c]	Adult, outbreak in restaurant
10	NV/8fIIa/68/US (NV)[d]	Adult, volunteer
11	UK2/1388/90/US (UK2-11)[e]	Adult, outbreak on cruise ship[f]
12	NV/2036/92/US[i] (UK2-12)[e]	Adult, outbreak on cruise ship[g]
13	UK2/1324/86/UK (UK2-13)[h]	Student, outbreak in school
14	UK3/10130/86/UK (UK3-14)[c]	Elderly, outbreak in geriatric hospital
15	UK3/SUMM/88/UK (UK3-15)[c]	Elderly, outbreak in geriatric hospital
16	UK3/9802/87/UK (UK3-16)[b]	Elderly, outbreak in geriatric hospital
17	UK3/12700/92/UK (UK3-17)[c]	Elderly, outbreak in geriatric hospital
18	UK3/1325/89/US (UK3-18)[b]	Adult, outbreak on cruise ship
19	UK4/5823/90/UK (UK4-19)[c]	Adult, food-borne outbreak
20	UK4/10336/90/UK (UK4-20)[c]	Elderly, outbreak in nursing home
21	UK4/1034/89/US (UK4-21)[b]	Adult, outbreak in restaurant
22	UK4/4880/86/US (UK4-22)[e]	Adult, outbreak on cruise ship

[a] Antigenic type/strain designation/year/country of detection.
[b] The sequence of the PCR product was determined in this study.
[c] The sequence corresponding to the PCR product region was determined by Ando et al. (2).
[d] From Jiang et al. (17).
[e] The sequence corresponding to the PCR product region was partially determined by Moe et al. (29) and completely determined in this study.
[f] From Herwaldt et al. (14).
[g] From Khan et al. (20).
[h] The sequence was not determined.
[i] The strain was classified as an NV type on the basis of its differential reactivity to NV and a reference strain for the UK2 type, UK2-8 (26), but was included in the UK2 type in this study.

MATERIALS AND METHODS

Virus samples. Fifteen stool specimens from adults and children involved in outbreaks and sporadic cases of gastroenteritis were collected by the Leeds and Taunton Public Health Service Laboratories between 1986 and 1992. Six stool specimens from adults with gastroenteritis were collected during outbreak investigations conducted in the United States between 1986 and 1992 by the Centers for Disease Control and Prevention (CDC). These 21 specimens were selected because they contained viruses that had been previously characterized by SPIEM (25–27). The 22 virus strains used in this study, including the prototype strain of NV from a volunteer, are identified by antigenic type, strain designation, year, and country of detection, and all were coded by antigenic type and consecutive number for the convenience of this study (Table 1).

RNA extraction. Ten to 25% stool homogenates were prepared in phosphate-buffered saline, mixed with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane, and clarified by centrifugation at about 500 × *g* for 5 min. A total of 0.1 ml of the stool extract was mixed with 0.9 ml of lysis buffer containing 4.6 M guanidinium thiocyanate, 20 mM EDTA, and 2% (wt/vol) Triton X-100. RNA in the mixture was extracted with phenol-chloroform and purified by adsorption to size-fractionated silicon dioxide particles (Sigma Chemical Co., St. Louis, Mo.) as previously described (3). The purified RNA was concentrated by ethanol precipitation, suspended in 20 μl of H_2O, and kept at −80°C until it was used in RT-PCR.

RT-PCR primers. Two sets of primers (G-1 and G-2) were designed for use in RT-PCR on the basis of nucleotide sequences of the RNA polymerase region from the genotype 1 and 2 SRSVs (2). Both G-1 and G-2 primer sets shared the same primer, SR33, for negative-strand cDNA synthesis. For positive-strand synthesis, the G-1 set contained three primers, SR48, SR50, and SR52, while the G-2 set contained one primer, SR46. Primer SR33 is located in the YGDD motif (17, 21) of the RNA polymerase region, and primers SR48, SR50, SR52, and SR46 are located at the same position between the GLPSG (17, 21) and YGDD motifs (Table 2). These primer sets delineated a predicted 123-bp product.

TABLE 2. Description of oligonucleotide primers and probes

Primer[a] or probe[b] set	Identification	Sequence	Polarity[c]	Location (nucleotides)[d]
Primer(s)				
G-1, G-2	SR33	tgt cac gat ctc atc atc acc	−	4856–4876
G-2	SR46	tgg aat tcc atc gcc cac tgg	+	4754–4773
G-1	SR48	gtg aac agc ata aat cac tgg	+	4754–4773
G-1	SR50	gtg aac agt ata aac cac tgg	+	4754–4773
G-1	SR52	gtg aac agt ata aac cat tgg	+	4754–4773
Probe				
P2-B	SR47d	atg tca ggg gac agg ttt gt	−	4804–4823
P2-A	SR61d	atg tcg ggg cct agt cct gt	−	4804–4823
P1-A	SR63d	aca tca gga gag tgc cca ct	−	4804–4823
P1-A	SR65d	aca tca ggt gat aag cca gt	−	4804–4823
P1-B	SR67d	aca tct ggt gag aga cct ga	−	4804–4823
P1-A	SR69d	aca tcg ggt gat agg cct gt	−	4804–4823

[a] Primer set including individual primer components.
[b] Probe set including individual probe component(s).
[c] −, negative; +, positive.
[d] Equivalent location within the NV genomic sequence (M87661).

Oligonucleotide probes. Six oligonucleotide probes labeled at the 5′ end with digoxigenin were designed on the basis of internal sequences at the same location in the 13 PCR products, as well as the sequences of the corresponding region previously reported for 17 SRSV strains (Table 2 and Fig. 2). The probes, SR63d, SR65d, and SR69d, were based on the sequences of four UK2 strains (UK2-8, UK2-9, UK2-11, and UK2-12), NV, and four previously reported strains. The P1-A probe set described below was composed of these three probes. Probe SR67d, which was subsequently redesignated the P1-B probe, was based on the sequences of two UK1 strains (UK1-6 and UK1-7), while SR61d (P2-A probe) was derived from those of seven SRSV strains, including four UK1 strains (UK1-1 to UK1-4). Probe SR47d (P2-B probe) was based on the sequences of five UK3 strains, four UK4 strains, and two reported SRSVs, including SMA (36). All primers and digoxigenin-labeled probes were synthesized in the CDC Biotechnology Core Facility (CDC, Atlanta, Ga.).

RT-PCR. RNA from each sample was tested by RT-PCR, using both primer sets G-1 and G-2 simultaneously in separate reactions. Before RT-PCR was performed, 1 μl of RNA was denatured at 95°C for 3 min in 30 μl of denaturation buffer containing 30 mM Tris-HCl (pH 9.0; at room temperature), 100 mM KCl, 4.5 mM $MgCl_2$, 0.2% (vol/vol) Triton X-100, 2 mM 2-mercaptoethanol, 1.67 mM (each) dATP, dCTP, dGTP, and dTTP, and 1 μM (each) primers SR33, SR48, SR50, and SR52 (G-1 primer set) or SR33 and SR46 (G-2 primer set). RT and PCR were carried out sequentially in one reaction tube by adding RT-PCR buffer to the denaturation buffer to make a 100-μl solution containing 15 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.25 mM $MgCl_2$, 0.1% Triton X-100, 1 mM 2-mercaptoethanol, 0.5 mM (each) dATP, dCTP, dGTP, and dTTP, 0.3 μM (each) G-1 and G-2 primers, 1 mM dithiothreitol, 40 U of human placental RNase inhibitor (Boehringer Mannheim Corp., Indianapolis, Ind.), 12 U of avian myeloblastosis virus super reverse transcriptase (Molecular Genetic Resources, Tampa, Fla.), and 5 U of AmpliTaq DNA polymerase (Applied Biosystems, Inc., Foster City, Calif.). The thermocycle format used in the RT-PCR was as follows: one cycle of reverse transcription at 42°C for 1 h, followed by denaturation at 94°C for 3 min; 40 amplification cycles with denaturation for 1 min at 94°C, annealing for 1 min 30 s at 50°C, and extension for 2 min at 60°C; and a final cycle of incubation at 72°C for 7 min. The amplification products were analyzed by electrophoresis in 3% agarose gels and visualized under UV illumination after staining with 0.5 μg of ethidium bromide per ml for 1 h.

Nucleotide sequencing. The 123-bp product from the RT-PCR was excised from the gel, extracted, and purified by using the Qiaex gel extraction kit (Qiagen Inc., Chatsworth, Calif.). Sequencing was carried out with both strands of the product with the Prism Ready Reaction Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc.) on an automated sequencer (Applied Biosystems model 373A DNA sequencing system). A consensus sequence for each PCR product was calculated with the Staden sequence analysis package (10), and a multiple alignment was generated by using the Pileup program of the Genetics Computer Group sequence analysis package (5). Phylogenetic analysis of the aligned sequences was done with the DNADIST, DNAML, and KITSCH modules of the PHYLIP package (8).

Southern hybridization using chemiluminescent detection. The PCR products were stained with ethidium bromide, denaturated for 1 h in a solution containing

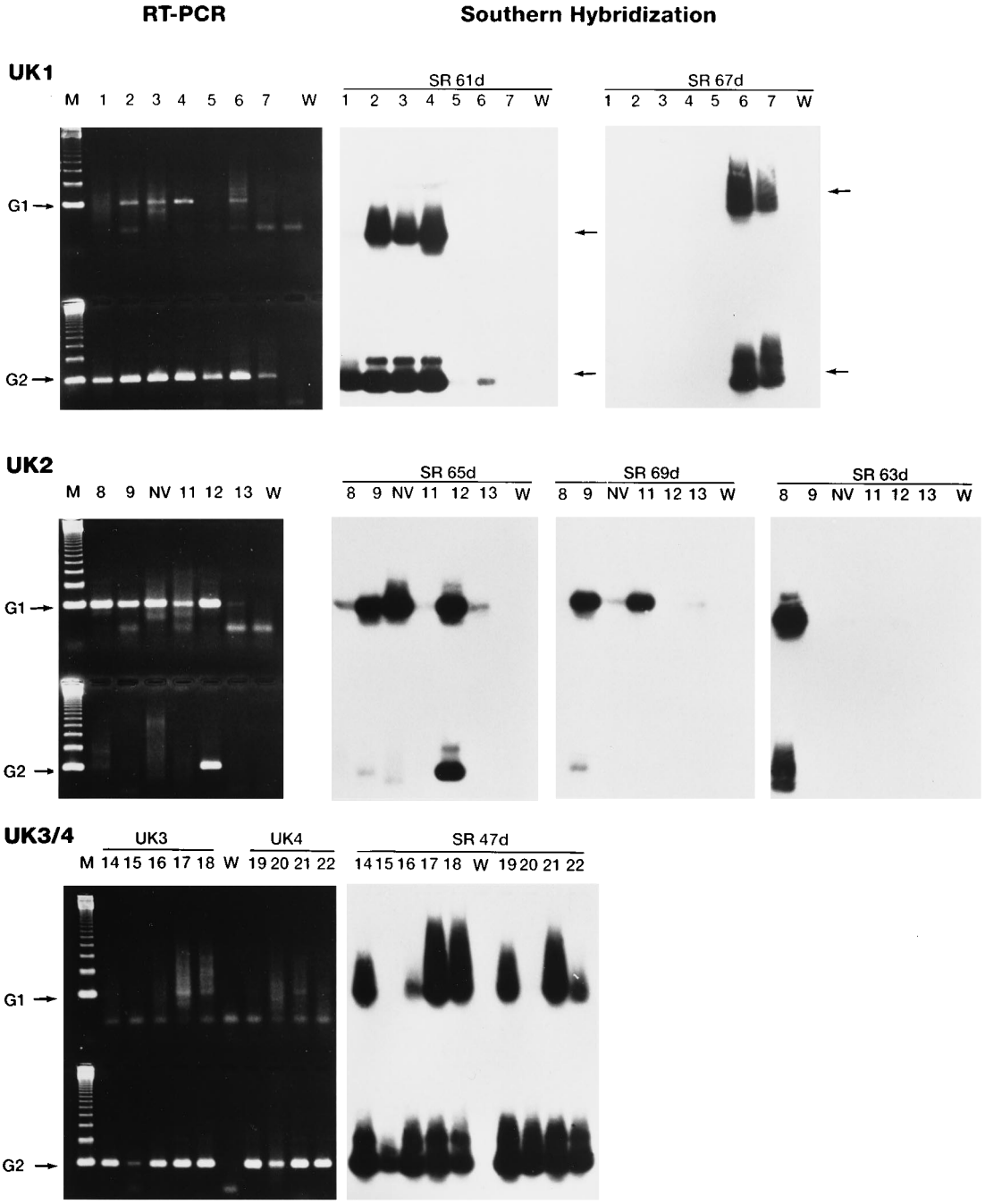

FIG. 1. Results of ethidium bromide staining and Southern hybridization of RT-PCR products from strains UK1 to UK4. The consecutive numbers on the wells of the gels and the blots correspond to the strain codes indicated in Table 1. M and W indicate 123-bp DNA marker (GIBCO BRL, Grand Island, N.Y.) and water used as the negative control for the RT-PCR, respectively. G-1 and G-2 indicate the primers, and the arrows indicate the 123-bp position of the expected molecular size of the RT-PCR product. The six oligonucleotide probes are placed on the top of the blots for the corresponding antigenic type of SRSVs.

0.5 N NaOH and 1.5 M NaCl and subsequently neutralized in 10× Tris-buffered saline (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5) for 30 min. The PCR products in the 3% agarose gel were then transferred overnight to a positively charged nylon membrane (Boehringer Mannheim Corp. Inc.) with 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), using a downward capillary transfer method as described previously (37), and immobilized by UV cross-linking with a Stratalinker model 1800 (Stratagene Cloning Systems, La Jolla, Calif.). Hybridization and chemiluminescent detection were carried out according to the protocols recommended by Boehringer Mannheim Corp., using blocking reagent, sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase, and Lumi-Phos 530 purchased from the same company. Briefly, the membrane was prehybridized in a solution containing 5× SSC, 0.1% *N*-laurylsarcosine, 0.02% sodium dodecyl sulfate (SDS), and 1.0% (wt/vol) blocking reagent at 58°C for 3 h and then hybridized in the prehybridization solution containing 10 pmol of digoxigenin-labeled probe per ml at 58°C overnight. For reaction of the hybridized digoxigenin-labeled probe with the antidigoxigenin-alkaline phosphatase conjugate, the membrane was pretreated with a blocking solution (100 mM Tris-HCl, 150 mM NaCl, 2% blocking reagent, pH 7.5) for 3 h at room temperature and incubated for 30 min at room temperature in the blocking solution containing the conjugate diluted 5,000-fold (150 mU/ml). For chemiluminescent detection, the membrane was incubated at 36°C for 30 min with Lumi-Phos 530 and exposed to X-ray film (Eastman Kodak Co., Rochester,

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            1                                                                           81   Number of Mismatches
                               SR69          ACA GGC CTA TCA CCC GAT GT                      SR69 SR65 SR63 SR67
    uk2-9   c.a a.c ..c t.g t.t ..c a.g t.t ..g .tc A.A ..C C.A ..A ..t ..T ..t a.a ..a t.c cag ..t .ac ..t ..t ..c ..t    1    2    6    4
   uk2-11   c.a a.c ..c t.g t.t ..c a.g t.t ..a .tc A.A ..C C.A ..A ..C ..T ..c a.a ..a t.c cag ..t .ac ..t ..t ..c ..t    0    3    7    4

                               SR65          ACT GGC TTA TCA CCT GAT GT
     ky89   a.a a.c ..c c.t t.t ..a t.g t.t ..g .ct A.T ..C T.A ..A ..T ..T ..g g.a ..g t.c atg ..a .ac ..c ..a ..c ..c    3    0    6    6
     1283   a.a a.c ..c c.t t.t ..a c.g t.t ..g .ct A.T ..C T.A ..A ..T ..T ..g g.g ..g t.c atg ..a .ac ..c ..a ..t ..c    3    0    6    6
       nv   a.a a.t ..t c.c t.t ..a c.g t.t ..g .cc A.T ..t T.A ..A ..T ..T ..g g.g ..a t.c atg ..a .at ..c ..a ..t ..t    4    1    6    5
   uk2-12   a.c c.a ..t c.a t.t ..a t.g t.a ..a .tc A.T ..C T.g ..c ..T ..T ..g a.a ..a t.a caa ..t .at ..c ..a ..t ..t    5    2    6    7

                               SR63          AGT GGG CAC TCT CCT GAT GT
    uk2-8   t.a a.t ..c c.t t.t ..c c.c t.t ..a .tt AcT ..G CtC ..T ..T ..T ..a a.a ..g t.a cag ..a .ac ..c ..a ..c ..t    5    4    2    5

                               SR67          TCA GGT CTC TCA CCA GAT GT
    uk1-6   a.t c.a ..t t.g a.t ..a a.g g.a ..a .tg T.A ..T C.C ..A ..A ..T ..t a.c ..a g.c cac ..c .gt ..t ..a ..t ..t    4    6    7    0
    uk1-7   a.t t.g ..c t.g a.t ..a a.g g.a ..a .tg T.A ..T C.C ..A ..A ..T ..t g.c ..a g.c cac ..c .gt ..c ..c ..t ..t    4    6    7    0
      dsv   a.t c.c ..c c.g t.t ..a t.g t.a ..a .tc a.a ..g C.C ..c ..A ..T ..g t.g ..g t.a cag ..g .at ..t ..c ..c ..t    5    6    5    4

      sov   t.a a.a ..t c.g t.c ..c c.t t.t ..a .tg A.T ..C c.g ..g ..a ..c ..t a.c ..a t.c atg ..a .at ..c ..t ..c ..t    5    5    7    6
Genotype1   -T- -T- AC- -T- -G- GC- -T- -C- GA- G-- -C- GG- -T- TC- CC- GA- GT- -T- CA- -C- --- TC- T-- TT- TC- TT- TA-

                               SR61          ACA GGA CTA GGC CCC GAC AT                      SR61 SR47
    uk1-1   t.g ..t ..t c.g ... ..c ..t ... ..a ..g ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..c a.g .ac ..t ..c ..t    0    7
    uk1-4   t.g ..t ..t c.g ... ..c ..t ... ..a ..g ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..c a.g .ac ..t ..c ..t    0    7
      925   t.g ..t ..t c.g ... ..c ..t ... ..a ..g ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..c a.g .ac ..t ..c ..t    0    7
      oth   t.g ..t ..t c.g ... ..c ..t ... ..a ..g ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..c a.g .ac ..t ..c ..t    0    7
    uk1-2   t.g ..t ..t t.g ... ..c ..t ... ..a ..g ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..c a.g .ac ..t ..c ..t    0    7
     tv24   t.g ..t ..t c.g ... ..c ..t ... ..a ..t ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..t a.g .ac ..t ..c ..t    0    7
    uk1-3   t.g ..c ..t c.a ... ..c ..t ... ..a ..g ..A GGA C.A GGC ..C ... ..c ..a ..a ... ..t ..c a.g .ac ..t ..c ..t    0    7

                               SR47          ACA AAC CTG TCC CCT GAC AT
   uk3-14   c.c ..c ..t c.c ... ..a ..c ... ..a ..t ..A AAC C.G TCC ..T ... ..t ..t ..g ... ..t ..c c.c .tc ..c ..c ..c    7    0
   uk3-16   c.c ..c ..t c.c ... ..a ..c ... ..a ..c ..A AAC C.G TCC ..T ... ..t ..t ..g ... ..t ..c c.c .tc ..c ..c ..c    7    0
   uk3-15   c.c ..c ..t c.c ... ..a ..c ... ..a ..t ..A AAC t.G TCC ..T ... ..c ..a ..g ... ..t ..c c.c .tc ..c ..t ..t    8    1
   uk3-18   c.c ..c ..t c.c ... ..a ..c ... ..a ..c ..A AAt t.G TCC ..T ... ..t ..a ..g ... ..t ..c c.c .tc ..c ..c ..t    8    2
   uk3-17   c.c ..c ..t c.c ... ..a ..c ... ..a ..t ..A AAC C.G TCC ..T ... ..c ..a ..g ... ..t ..c c.c .tt ..c ..c ..t    7    0
      brv   c.c ..c ..t c.c ... ..a ..c ... ..a ..t ..A AAC C.G TCC ..T ... ..c ..a ..g ... ..t ..c c.c .tt ..c ..c ..t    7    0
   uk4-19   c.c ..c ..t c.c ... ..a ..c ... ..g ..t ..A AAC C.G TCC ..T ... ..t ..c ..g ... ..t ..c c.c .tt ..c ..c ..t    7    0
   uk4-20   c.c ..c ..t c.c ... ..a ..c ... ..g ..t ..A AAC C.G TCC ..T ... ..t ..c ..g ... ..t ..c c.c .tt ..c ..c ..t    7    0
   uk4-21   c.c ..c ..t c.c ... ..a ..c ... ..g ..t ..A AAC C.G TCC ..T ... ..t ..c ..g ... ..t ..c c.c .tt ..c ..c ..t    7    0
      sma   c.c ..c ..a c.c ... ..a ..a ... ..a ..c ..A AAC C.G gCt ..T ... ..c ..a ..a ... ..c ..c t.g .tc ..t ..c ..t    7    2
   uk4-22   c.c ..c ..c c.t ... ..a ..t ... ..a ..c ..g AAC C.G TCC ..T ... ..c ..a ..a ... ..t ..t c.a .tt ..t ..c ..t    8    1
    uk1-5   c.t ..c ..c c.t ... ..g ..t ... ..g ..c ..A AAC C.G TCC ..T ... ..c ..c ..a ... ..t ..t c.c .tt ..t ..c ..t    7    0
Genotype2   -T- CT- AC- -T- TGT GC- CT- TCT GA- GT- AC- --- -T- --- CC- GAC AT- AT- CA- GCT AA- TC- -T- T-- TC- TT- TA-


       G1   -T- -T- AC- -T- -G- GC- -T- -C- GA- G-- -C- GG- -T- TC- CC- GA- GT- -T- CA- -C- --- TC- T-- TT- TC- TT- TA-
       G2   -T- CT- AC- -T- TGT GC- CT- TCT GA- GT- AC- --- -T- --- CC- GAC AT- AT- CA- GCT AA- TC- -T- T-- TC- TT- TA-
Consensus   -T- -T- AC- -T- -G- GC- -T- -C- GA- G-- -C- --- -T- --- CC- GA- -T- -T- CA- -C- --- TC- --- T-- TC- TT- TA-
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FIG. 2. Alignment of the nucleotide sequence of the 81-bp unique region (excluding the two primer regions). The number 1 corresponds to nucleotide 4774 in the genome of NV (17). The sequences of 30 strains are separated into two genotypes: the upper 11 strains belong to genotype 1, and the lower 19 strains belong to genotype 2. The sequences in each genotype are arranged in clusters according to the results of the phylogenetic analyses (Fig. 3) and are compared with the sequences complementary to the oligonucleotide probes, which were derived from the sequences in the corresponding clusters. The number of sequence mismatches between the probes and the SRSVs are indicated at the right. The dots represent sequence identity with the consensus sequence for each genotype, and the lowercase letters represent sequence variation between the consensus sequences and those of the strains used in this study. Within the probe region, uppercase letters are positions of agreement with the indicated probe. GenBank accession numbers for the sequences used for the comparison were L23828 (ky89), L23832 (1283), M87661 (NV), V04469 (DSV), L07418 (SOV), L23826 (925), L23830 (OTH), U02030 (TV24), X76716 (BRV), L23831 (SMA), and U07611 (HWA) (23). An antigen detection ELISA based on the recombinant NV indicated that ky89 and 1283 detected in Japan were antigenically related to NV, while 925 (UK) and OTH (JPN), as well as the prototype strain of SMA and Toronto virus (TV24), were not related to NV (36, 24). Desert Shield virus (DSV) was antigenically related to but distinguishable from NV as defined by the ELISA based on recombinant NV (22). Southampton virus (SOV) was previously classified as SMA type in antigenicity (21), but its antigenicity has remained unclear because the virus was typed by immune electron microscopy with patient sera which were not fully characterized. Information about antigenic characteristics of Bristol virus (BRV) was not available.

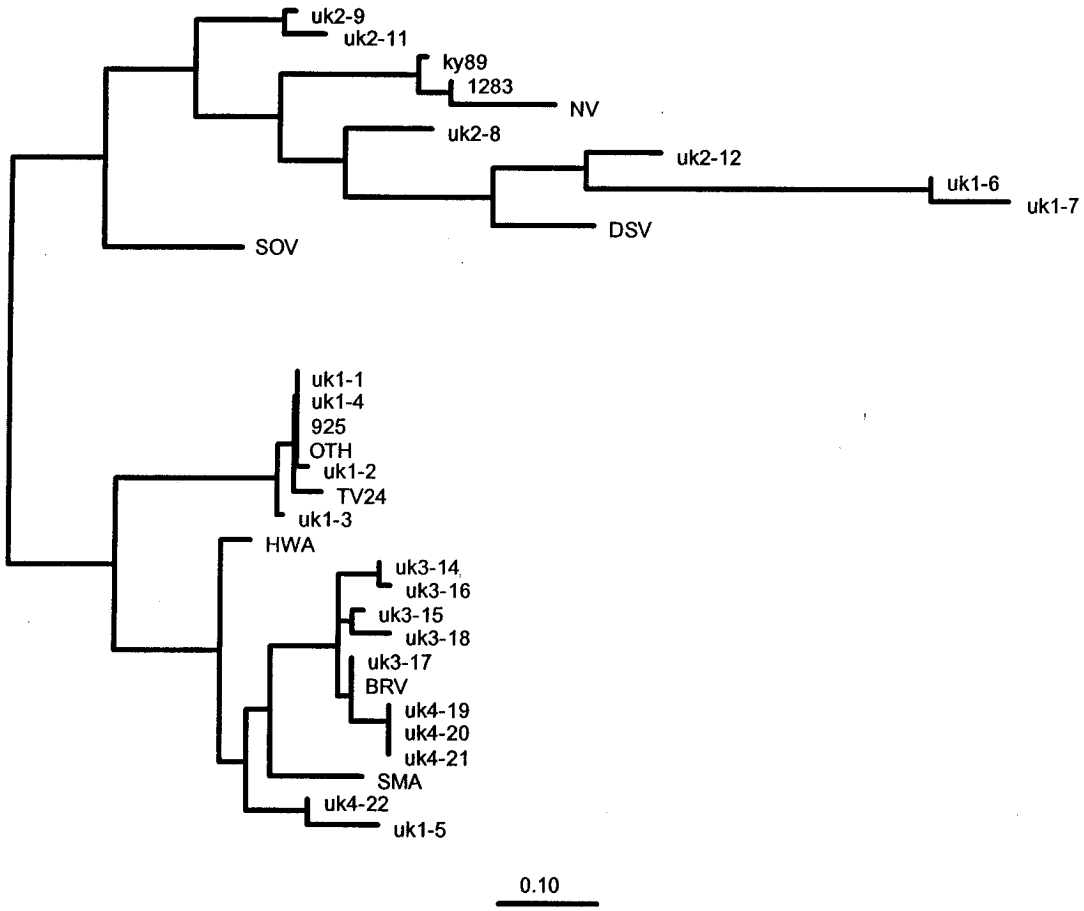

FIG. 3. Dendogram of predicted phylogenetic relationship among 30 SRSV strains used in Fig. 2 and HWA (23). The length of the abscissa to the connecting node is proportional to the genetic distance between sequences. DSV, Desert Shield virus; SOV, Southampton virus; BRV, Bristol virus.

N.Y.) for 5 min at room temperature. The membrane was stripped by incubation at 60°C for 10 min and 37°C for 20 min in a solution containing 0.2 N NaOH and 0.1% SDS, rinsed with 2× SSC buffer, UV cross-linked again, and kept at 4°C until it was used for reprobing. For reprobing the membrane, the procedures for hybridization were followed beginning at the prehybridization step.

Restriction endonuclease analysis. For each sample, the PCR products from three to five reaction tubes were pooled, extracted with phenol-chloroform, and concentrated by ethanol precipitation. The product was resuspended in 25 μl of H_2O, and the concentration was spectrophotometrically determined. The product was adjusted in concentration to 200 ng per reaction (22 μl) and subjected for 3 h to 10 U of *Bfa*I, 10 U of *Ban*II, and 20 U of *Stu*I at 37°C or to 10 U of *Bsa*I at 55°C. The enzymes and 10× buffers used were purchased from New England BioLabs, Inc. (Beverly, Mass.). The digestion products were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining and Southern hybridization as described above.

RESULTS

RT-PCR. For the 22 SRSV strains examined, the primer set that resulted in the greater amount of PCR product as estimated by ethidium bromide staining correlated well with the antigenic type as determined by SPIEM (Fig. 1). In general, the G-1 primers preferentially amplified the UK2 strains, while G-2 primers preferentially amplified the UK1, UK3, and UK4 strains. For the seven UK1 strains, RT-PCR with the G-2 primers yielded products from all strains, including six for which the amount of product was high, while the G-1 primer set yielded products with moderate to weak intensity from four of seven strains examined. Comparison of the G-1 and G-2 products obtained from the same strain clearly indicated that the G-2 primers resulted in a greater amount of product than did the G-1 primers in all four strains detectable by both primer sets (Fig. 1, upper row, left column). For six viruses of the UK2 type, the G-1 primers yielded intense products from five strains and a weaker product from the sixth, while the G-2 primers yielded an intense product from only one strain, a product with an intensity comparable to that of the G-1 product obtained from the same strain (Fig. 1, middle row, left column). As for the UK3 and UK4 strains, the G-2 primers yielded intense products from the five UK3 and four UK4 strains examined, while the G-1 primers yielded products with very weak intensity from two UK3 strains and one UK4 strain (Fig. 1, lower row, left column).

Nucleotide sequencing. The 13 nucleotide sequences determined in this study, which included those of 2 G-1 products of UK2 strains and 11 G-2 products of UK1, UK3, and UK4 strains (Table 1), were compared with the sequences reported for 17 SRSVs, including seven strains used in this study (2). The alignments of the nucleotide sequence of the unique 81-bp region (excluding the two amplification primers) demonstrated considerable nucleotide conservation among the SRSV strains: 33 (41%) of the 81 nucleotides were completely conserved among all 30 strains. These conserved nucleotides were distributed throughout the sequence, except in the region corresponding to nucleotides 33 to 42, where the sequences were more variable (Fig. 2).

The sequences were further analyzed by using a maximum-likelihood algorithm (DNAML) to estimate the phylogenetic relationships among the various strains (Fig. 3). The strains were separated into two distinct lineages, consistent with previous results (2). The pairwise nucleotide identity between the two genotypes ranged from 54 to 73%. Within genotype 1, the pairwise identity ranged from 59 to 95%, while within genotype 2 the range was 70 to 100%.

In general, the primer set which yielded the greater amount of product was in good agreement with the genotype with which the sequence clustered. However, strains UK1-6 and UK1-7, which were preferentially amplified with G-2 primers, were placed in genotype 1 rather than genotype 2. In addition, strain UK1-5 clustered with UK3 and UK4 strains but not with other UK1 strains. On the basis of the sequences of a 20-nucleotide region characteristic to each cluster, which included variable residues 33 to 42 described above, we designed six digoxigenin-labeled probes for Southern hybridization (Table 2).

Southern hybridization. The results of ethidium bromide staining were confirmed by Southern hybridization. The G-1 products of all six UK-2 strains hybridized with one or two of the probes SR65d, SR69d, and SR63d (Fig. 1, lanes 8 to 13). The G-2 products of six UK1 strains hybridized with SR61d or SR67d (Fig. 1, lanes 1 to 7), and those of all five UK3 and four UK4 strains hybridized with SR47d (Fig. 1, lanes 14 to 22). Cross-priming with G-1 primers was detected by the hybridization in UK1 (Fig. 1, lanes 2 to 4, 6, and 7), UK3 (Fig. 1, lanes 14 and 16 to 18), and UK4 (Fig. 1, lanes 19, 21, and 22) strains, and cross-priming with G-2 primers was seen in UK2 strains (Fig. 1, lanes 8, 9, and 12). In addition, cross-hybridization with two probes was observed in one UK1 (Fig. 1, lane 6) and two UK2 (Fig. 1, lanes 8 and 9) strains. However, neither SR61d nor SR67d hybridized with the G-2 product from the UK1-5 strain, although its intensity by ethidium bromide staining was relatively strong (Fig. 1, lane 5).

On the basis of the phylogenetic clustering of the nucleotide sequences, we pooled SR65d, SR69d, and SR63d to make probe set P1-A, and the individual probes SR67d, SR61d, and SR47d were redesignated probe sets P1-B, P2-A, and P2-B, respectively. We used these four probe sets to differentiate the G-1 and G-2 products of RT-PCR according to the antigenic type of the template SRSV (Fig. 4A). Probe P1-A hybridized with G-1 products of all six UK2 strains, and no cross-hybridization was observed in SRSVs of other antigenic types (Fig. 4B). Probe P1-B hybridized with the G-2 products of two UK1 strains (UK1-6 and UK1-7) and weakly cross-hybridized with the G-1 products of two of the UK2 strains, UK2-8 and UK2-9 (Fig. 4C). Probe P2-A hybridized with the G-2 products of four of seven UK1 strains examined (the data of two positive strains, UK1-1 and UK1-2, were not shown due to lack of space in the agarose gel) (Fig. 4D). As for probe P2-B, the G-2 products of all UK3 and UK4 strains hybridized with this probe (Fig. 4E). This probe hybridized with the G-2 product of UK1-5 strain (Fig. 4E, lane 5), which did not hybridize with probes P1-B and P2-A. Since strain UK1-5 showed nucleotide sequence similarity to the UK3 and UK4 strains, and its G-2 product specifically hybridized with P2-B primer, we retested this strain by SPIEM. The results indicated cross-reactivity of this strain with the UK1 and UK4 reference typing sera (data not shown).

Restriction endonuclease analysis. Since the G-1 products of UK2-8 and UK2-9 strains cross-hybridized with probe set P1-B, we analyzed them with four restriction endonucleases (*Bfa*I, *Bsa*I, *Ban*II, and *Stu*I). *Ban*II and *Stu*I completely digested the UK2-8 and UK2-9 products, respectively, detectable by both P1-A and P1-B probes, while digestion of *Bfa*I and *Bsa*I did not affect the amount of these products detectable by these probes. In contrast, *Bfa*I and *Bsa*I digested the G-2 product of UK1-3 and UK1-7 strains, respectively, which were used as the strains representing two clusters of the UK1 type described above (Fig. 3), while *Ban*II and *Stu*I did not digest the products (data not shown). These results indicated that cross-hybridization of UK2-8 and UK2-9 strains with the P2-B probe was not caused by SRSV sharing the sequence with the UK1 strains used in this study. As for cross-hybridization of the G-1 product from the UK1-6 strain with probes SR61d and SR67d (Fig. 1, lane 6), the weak hybridization signal by SR61d

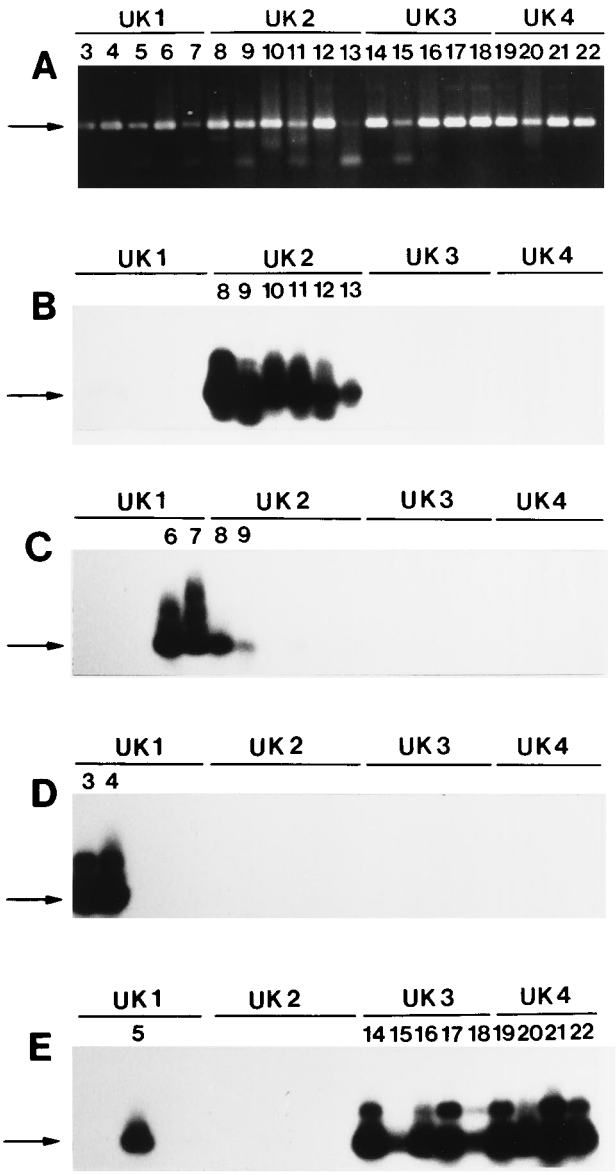

FIG. 4. Results of differential detection of antigenically distinct SRSVs by RT-PCR and Southern hybridization. The G-1 products of the RT-PCR from six UK2 strains and the G-2 products from five UK1, five UK3, and four UK4 strains (A) were differentially detected by Southern hybridization, using probe sets P1-A (B), P1-B (C), P2-A (D), and P2-B (E), respectively. The consecutive numbers on the wells of the gel (panel A) correspond to the strain codes (Table 1), as described in the legend to Fig. 1. The strains positive for an individual probe were highlighted by the numbers on the wells of the blot.

TABLE 3. Interim scheme for differential detection of SRSVs with distinct antigenic type by RT-PCR and Southern hybridization

PCR primer	Probe	UK type (prototype)
G-1	P1-A	UK2 (NV)
G-2	P1-B	UK1 (?)
	P2-A	UK1 (TNA)[a]
	P2-B	UK3/4 (HWA/SMA)

[a] TNA, Taunton agent.

was not reproduced in subsequent experiments (data not shown).

DISCUSSION

In this report, we describe a method for the detection and differentiation of antigenically distinct SRSVs by using RT-PCR and Southern hybridization. The primers used in the RT-PCR were designed on the basis of our recent finding that various SRSVs antigenically related to NV, HWA, and SMA could be classified into two genotypes on the basis of the sequences of a 166-base region in the RNA polymerase gene (2). The internal oligonucleotide probes used in the Southern hybridization were designed on the basis of the nucleotide sequences of 30 SRSV strains, including those of 21 strains used in this study. To verify the reactivity and specificity of the primers and probes, we used a total of 22 antigenically distinct SRSVs, including seven UK1, six UK2, five UK3, and four UK4 strains, which were previously characterized by SPIEM.

The results of ethidium bromide staining of the RT-PCR products, as well as those of the Southern hybridization, have proven that the primers are broadly reactive with many antigenically distinct SRSVs since all of the 22 SRSV strains examined could be detected with either the G-1 or the G-2 primer set. The G-1 and G-2 primers selectively amplified members of the G-1 and G-2 genotypes, respectively, although cross-priming was observed in some of the strains. Such cross-priming may be explained by the fact that the G-1 and G-2 sets contain the same primer (SR33) of negative polarity and the primers of positive polarity which share 10 of 21 nucleotides, including five of the first six 3′-end nucleotides (Table 2) (31). In our differentiation system, however, disadvantages related to cross-priming in the RT-PCR were well overcome by the Southern hybridization with internal oligonucleotide probes that selectively hybridized with the RT-PCR products from the UK2, two of UK1, four of the UK1, and both the UK3 and the UK4 strains. In addition, the phylogenetic analysis, which was carried out on the sequences of the RNA polymerase region from the 30 SRSV strains, indicated several important aspects regarding the relationship between the nucleotide sequence of this region and the antigenic type of SRSVs. First, although the analysis was based on a region of only 81 nucleotides, less than half the size used in the previous analysis (2), the SRSVs of UK2, UK3, and UK4 antigenic types could still be classified into two distinct groups. Second, the UK2 type showed much greater nucleotide diversity than did UK3 and UK4 types. Third, although all seven UK1 strains were preferentially detected by G-2 primers, which were originally designed to detect members of UK3 and UK4 strains, they appeared to belong to lineages distinct from that of the UK3 and UK4 strains. Two UK1 strains (UK1-6 and UK1-7) were placed in the phylogenetic lineage of genotype 1, more closely related to the UK2 strains than to the remaining UK1 strains. Of the remaining five UK1 strains, which were placed in a distinct branch of the genotype 2 lineage, four strains (UK1-1 to UK1-4) clustered with Toronto virus (TV24) and two Japanese strains (OTH and 925) (24, 36). The last UK1 strain (UK1-5) was clustered with one of the UK4 strains (UK4-22).

The results of the phylogenetic analysis of the UK1 strains were consistent with the observation that the RT-PCR products of the UK1 strains in the three clusters described above separately hybridized with probe sets P2-A (SR67d), P1-B (SR61d), and P2-B (SR47d), respectively. It is possible that the genetic heterogeneity observed among the UK1 strains was, at least in part (as shown for UK1-5 upon retesting by SPIEM), a result of the potential for artifacts of antigenic typing by SPIEM being dependent on the use of human reagents whose specificity has not been fully characterized. Alternatively, the nucleotide sequences of the 123-base region analyzed phylogenetically may not correspond to the antigenic features in some of the UK1 strains. Future studies using more specific reagents based on expressed capsid proteins may help to reconcile the antigenic and genetic classifications of the UK1 strains.

In summary, the results of this study reinforce our previous hypothesis that SRSVs of UK2, UK3, and UK4 antigenic types (2) belong to two genotypes that can now be distinguished by RT-PCR. We could further differentiate viruses from UK1 to UK4 types into four subgroups by the combined use of RT-PCR and Southern hybridization (Table 3). The availability of these assays used together should facilitate study of the characterization of the antigenic and genetic relationships among SRSVs, as well as study of the epidemiology and transmission of this group of viruses.

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