Human enteric members of the family *Caliciviridae* include small round-structured viruses (SRSVs) and the morphologically classical human calciviruses. SRSVs, also known as Norwalk-like viruses, are important etiologic agents associated with food- and waterborne outbreaks of acute non-bacterial gastroenteritis (12, 20). Norwalk virus (NV) is the recognized prototype of the SRSVs and was first visualized and identified in 1972 by immune electron microscopy (EM) (22). Since then progress in investigating the role of SRSVs in outbreaks of gastroenteritis and in the characterization of Norwalk-like viruses has been significantly hindered by the lack of antigen to produce serological assays, the inability to propagate the virus in cell culture, and the absence of an appropriate animal model (20).

However, since the success in 1990 of Jiang et al. (14) in cloning the Norwalk virus genome, and the subsequent expression of recombinant Norwalk capsid protein in baculovirus, which has provided the material for producing enzyme immunoassays (EIAs) (17), numerous large-scale epidemiological studies have been conducted. Seroprevalence studies of NV indicate a high incidence of exposure and infection in most countries where studies have been undertaken. Epidemiological studies conducted in the 1970s using radioimmune and hemagglutination assays determined that the prevalence of anti-NV antibodies appears to be low in infants and young children but increases significantly during adulthood in developed countries (4, 11, 21). In contrast, in developing countries NV infection starts in early childhood (<24 months) (3, 6).

The genome sequences of NV and other strains of SRSVs have shown that the single-stranded viral RNA genome of SRSVs is approximately 7.6 kb in length (8, 27) and has three open reading frames (ORFs) which are characteristic of the *Caliciviridae* (19, 23). The largest ORF at the 5′ end encodes the viral enzymes, including the RNA-dependent RNA polymerase, while ORF 2 encodes the single structural protein, the capsid protein of 59 to 72 kDa (10, 27).

Amino acid and nucleotide sequence analysis of a region of the RNA polymerase from many isolates of SRSVs has led to the distinction of two genetic groups among the viruses infecting humans (2, 9, 36). Sequencing studies of the capsid genes of SRSVs have further confirmed this observation (2, 26, 36).

NV is the prototype of genogroup I, which includes Southampton virus, and is phylogenetically distinct from viruses of genogroup II, which includes the prototype Snow Mountain agent, Bristol, Hawaii, Toronto, and Mexico viruses (2, 25, 26).

SRSVs have now been identified as the etiologic agent of a number of gastroenteritis outbreaks which were previously associated with an unknown or unidentified agent (9, 12, 16, 31). In a previous study at Ga-Rankuwa Hospital, Ga-Rankuwa, South Africa, as many as 40% of the cases of diarrhea were associated with an unidentifiable etiologic agent (35).

Since antibody EIA systems with either recombinant NV (rNV) or recombinant Mexico virus (rMxV) capsid protein have been developed and are now available in this laboratory, we initiated this study at Ga-Rankuwa (i) to determine the seroprevalence of NV and MxV in adults, (ii) to determine the pattern of antibody acquisition to these two viruses in both children and adults. Although the viral antigens are not highly prevalent in diarrheal stools, it was determined by the two assays for NV and MxV that children are, nevertheless, infected early in life.

**MATERIALS AND METHODS**

**Seroprevalence study.** (i) Serum samples. Sera were collected from 488 individuals ranging in age from 3 to 87 years, forming a family-based cohort from Ga-Rankuwa of which 215 were male and 273 were female. A further 686 sera...
were collected from patients attending the antenatal clinic. These patients were all female and were 15 to 49 years of age. The third cohort consisted of sera collected from 264 infants and young children, from 0 to 84 months in age, who presented with symptoms other than gastroenteritis. Of these 264 sera, 143 were from males, and the remaining 121 sera were from females.

All cohorts were derived from the population of Ga-Rankuwa, which lies northwest of Pretoria and which is inhabited by between 75,000 and 100,000 persons. Ga-Rankuwa is in essence a developing urban area with reasonable infrastructures including streetlights, telephones, water and electricity supplies to most but not all homes, sewers, and tarmacked roads (28).

(ii) Recombinant EIAs to detect antibodies to NV and MxV in sera. All serum samples were analyzed for the presence of antibodies to NV and MxV by the EIA techniques previously described (13, 33). Diluted test sera were added to wells of microtiter plates (Immulon-2, Dynatech) coated with baculovirus-expressed rNV or rMxV capsid protein. An alkaline phosphatase-conjugated goat anti-human immunoglobulin G was used as detector antibody, and TMB was used as the substrate. After development of the enzymatic reaction, the absorbance was read at 450 nm. Test reactions with absorbance values greater than twice the mean of the negative controls and ± 0.20 were considered positive.

Examination of stool specimens. (i) Stool specimens. Stool specimens were collected from 276 infants and young children (160 males and 116 females) ranging from 3 days to 54 months in age who presented with diarrhea at Ga-Rankuwa Hospital. The children were seen as outpatients at Ga-Rankuwa Hospital and presented with acute infantile nonbacterial diarrhea with moderate to severe dehydration.

(ii) Recombinant EIA to detect NV and MxV antigens in stool specimens. Stool specimens were tested, essentially as previously described, for the presence of NV and MxV antigens by using recombinant EIAs reported to be specific for these two antigenic types (13, 18). Duplicate wells were coated with a 1:10,000 dilution of either rabbit pre- or postsera, specific for either NV or MxV. Ten percent stool suspensions were then added to the wells. Guinea pig anti-NV or anti-MxV hyperimmune sera was used, together with a goat anti-guinea pig peroxidase-conjugated immunoglobulin G and TMB for the detection of the reaction. The net absorbance value for each sample at 450 nm was determined as the value in the antigen-coated test well (P) minus the value in the antigen-negative well (N). Results with a net absorbance value of ±0.20 and a P/N value of ≥2.0 were considered positive.

(iii) EM examination of stool specimens. One hundred-thirty stool specimens were randomly selected and examined by negative-contrast EM as described previously (35) for the presence of characteristic SRSVs, which are 33 to 35 nm in diameter. The specimens were examined under an electron microscope (JOEL 100 CX) at a magnification of ×40,000.

RT-PCR amplification of viral RNA. (i) RNA extraction from stools. Twelve stool samples were selected on the basis of the EM results for follow-up testing by reverse transcription (RT)-PCR. A 10 to 20% stool suspension of each of the 12 samples was made in distilled water. Three hundred microliters was then used for the extraction of the viral RNA by the polyelectrolyte glycolated trimethylammonium bromide method described by Jiang et al. (16). The final RNA extract was resuspended in 20 μl of water.

(ii) Detection of virus in stool specimens by RT-PCR. Five microliters of extracted RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega), essentially as described previously (18). The primer pair 35-36, directed against a region of the RNA-dependent RNA polymerase gene, was used (36). The PCR-amplified products were then analyzed on a 1% agarose gel and visualized by ethidium bromide staining and UV illumination.

(iii) Sequencing of RNA-dependent RNA polymerase region. The nucleic acid sequence of the 470-bp product of the RT-PCR-amplified RNA-dependent RNA polymerase region of the Ga-Rankuwa SRSVs (represented by SRSV-GR149/93/RSA) was determined by the dideoxynucleotide chain termination method with the Sequenase PCR Product Sequencing Kit (U. S. Biologicals, Cleveland, Ohio).

RESULTS

EIA for detection of antibodies to NV and MxV. Results obtained by EIA for the prevalence of antibodies to both NV and MxV revealed seroprevalences of 98% for NV and 99% for MxV for the family-based cohort in Ga-Rankuwa, while for the antenatal cohort at Ga-Rankuwa Hospital, 96% had antibodies to both NV and MxV.

Age of acquisition of antibodies to NV and MxV. The patterns for the age of acquisition of antibodies to NV and MxV were shown to be quite similar (Fig. 1). As expected the neonates showed antibody levels similar to those found in the mothers. Remarkably, the antibody levels dropped rapidly initially, before there was a steady increase in the prevalence of antibodies to both of these viruses, and by 48 months all infants had been exposed and had developed antibodies (Fig. 1).
EIA to detect NV and MxV antigens in stool specimens. Only 5 of 276 (1.8%) of the stool specimens collected from children with gastroenteritis were positive for antigen to NV. A slightly higher incidence of MxV, 12 of 275 (4.3%), was detected in the stool specimens tested.

EM examination of stool specimens. Of the 130 stool specimens examined, 12 (9.2%) had identifiable SRSVs as determined by negative-contrast EM. The particles were approximately 33 nm in diameter.

RT-PCR amplification of viral RNA in stool specimens. Three of the 12 (25%) EM-positive stool specimens were positive by RT-PCR with primer pair 35-36. The PCR-amplified products were predicted to be approximately 470 bp in size.

Sequencing of RNA-dependent RNA polymerase region. The deduced amino acid sequences of all three strains were identical. The representative strain (SRSV-GR149/93/RSA) was aligned with published sequences for viruses of the two genogroups of human caliciviruses and four other South African SRSVs (Fig. 2), and a dendrogram was constructed by using the CLUSTAL program (version 6.8). The Ga-Rankuwa SRSVs, represented by SRSV-GR149/93/RSA, were found to cluster with MxV and the other related South African SRSVs (Fig. 3).

DISCUSSION

SRSVs have been identified as the causative agent of a number of outbreaks of gastroenteritis and are now recognized as an important etiologic agent of diarrheal illness worldwide. In a previous study conducted at Ga-Rankuwa Hospital, a large number of episodes of diarrhea were associated with an unidentifiable agent (35). Our study has determined that SRSVs are prevalent in the local population and may account for cases of diarrhea of previous unknown cause.

In this study we have determined that infections with both genogroups of the human caliciviruses, namely Norwalk-like (genogroup I) and Snow Mountain-like viruses (genogroup II), were highly prevalent in a family-based cohort and in an antenatal clinic cohort. The antibody seroprevalences of both viruses were greater than 95% in both cohorts. This is somewhat different from the reported 50 to 80% seroprevalences to NV for the United Kingdom and the United States (11, 21). However, the seroprevalence figures in our study population are similar to those in other developing communities such as in Mexico and Southeast Asia and among the Australian Aborigines (16, 31, 34).

Antibodies to NV and MxV are apparently acquired by 24 to 48 months of age in children from Ga-Rankuwa. The pattern for the acquisition of antibodies is once again different from that of developed countries like the United Kingdom, the United States, and Japan where antibodies are generally only acquired by children of school age (11, 21, 31, 34). However, it is similar to that seen in developing communities in Kuwait, Bangladesh, and Mexico and among the Australian Aborigines where children acquire the infection at a younger age (approximately 2 years) (7, 11, 16, 34).

This study has also shown that NV and MxV infections are acquired early in life and appear to be important causative agents of infantile diarrhea in Ga-Rankuwa. NV antibodies appear to have been acquired a little earlier on in life than antibodies to MxV. This is in contrast to a study in London, United Kingdom (32), where it was determined that infection with MxV occurred earlier than NV infection.

Maternal antibody in the infant was shown by Parker et al. to wane over time, and natural infection, which correlated with an increase in the overall optical density (OD) values, was re-
ported to occur in children more than 2 years of age in London, United Kingdom (34). By contrast, in our study natural infection with NV (and MxV) appears to occur during the first 12 months of age, as shown by a broader and higher range in the overall OD values (data not shown). Maternal antibodies to both NV and MxV were detected in the first 9 months of life, probably due to the high sensitivity of the tests as described previously (32). However, by the criteria of Parker et al. (34), the increase in the OD values in this study after 4 months of age suggests a natural exposure and antibody response in the infants and not the presence of passively acquired maternal antibodies.

It was interesting to note that although antibodies to NV appeared more rapidly than antibodies to MxV, genogroup II (Snow Mountain agent-like) viruses were more common in the stools from infants with gastroenteritis, although the number of stools examined was small. A similar observation was made in a recent study conducted in South Africa in which Norwalk-like viruses were more commonly associated with adult gastroenteritis, while Mx-like viruses were associated with pediatric gastroenteritis (38). Furthermore, both studies conducted in South Africa have determined NV to be present in various cohorts, unlike a study in the United Kingdom which reports that “Norwalk virus is no longer circulating” (5).

The deduced amino acid sequence of the amplified region of the RNA polymerase gene from our SRSVs, represented by SRSV-GR149/93/RSA, was identical to that of other SRSVs associated with pediatric gastroenteritis in South African children (38). However, several viruses identified by recombinant EIA and EM did not react in the RT-PCR in our study. Due to the known genetic diversity within the genogroups of SRSVs a low detection rate by RT-PCR may be expected (1, 8, 37). The detection rate of viral antigen in stool specimens in our population (38) is in agreement with previous reports. However, several viruses identified by recombinant EIA and EM did not react in the RT-PCR in our study. Due to the known genetic diversity within the genogroups of SRSVs a low detection rate by RT-PCR may be expected (1, 8, 37). The detection rate of viral antigen in stool specimens in our population (38) is in agreement with previous studies.

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