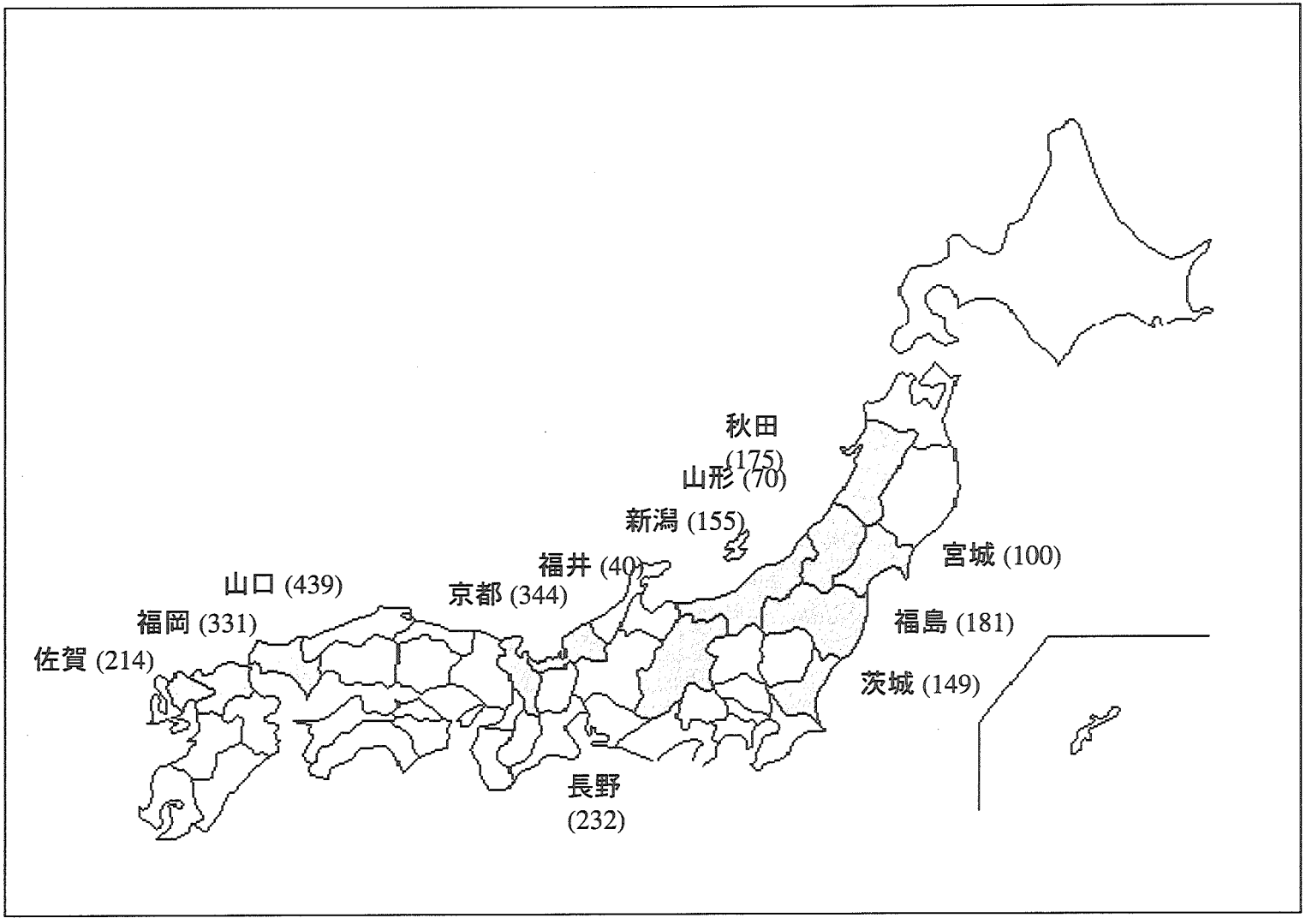

図１．採取した血清の地域とその数

表１．男女別HAV抗体陽性率

	男性	女性	計
陽性数/検体数	158 / 1242	139 / 1188	297 / 2430
陽性率(%)	12.7	11.7	12.2

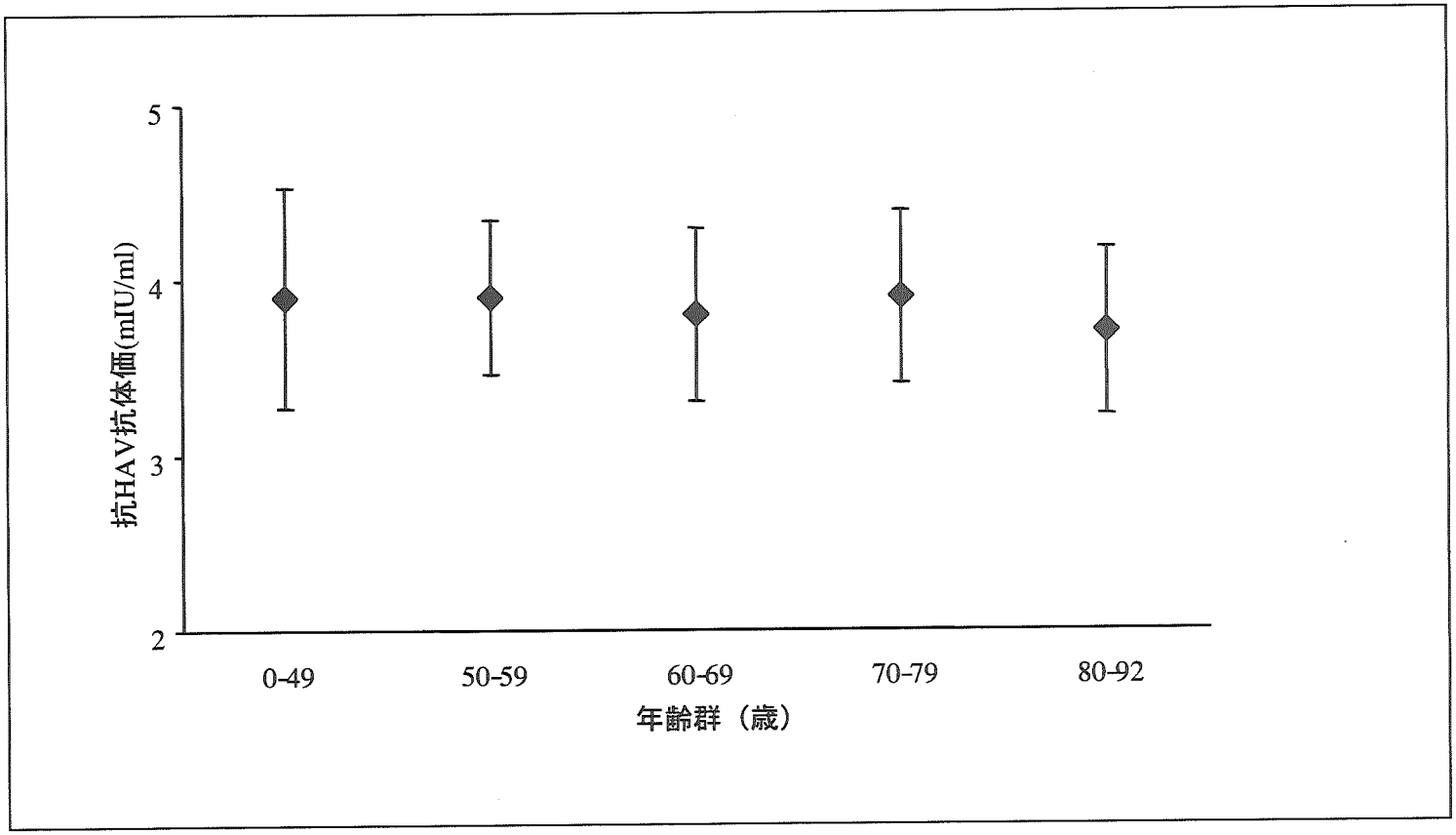

図２．年齢群毎の抗HAV抗体価、誤差線は1SDを示す。

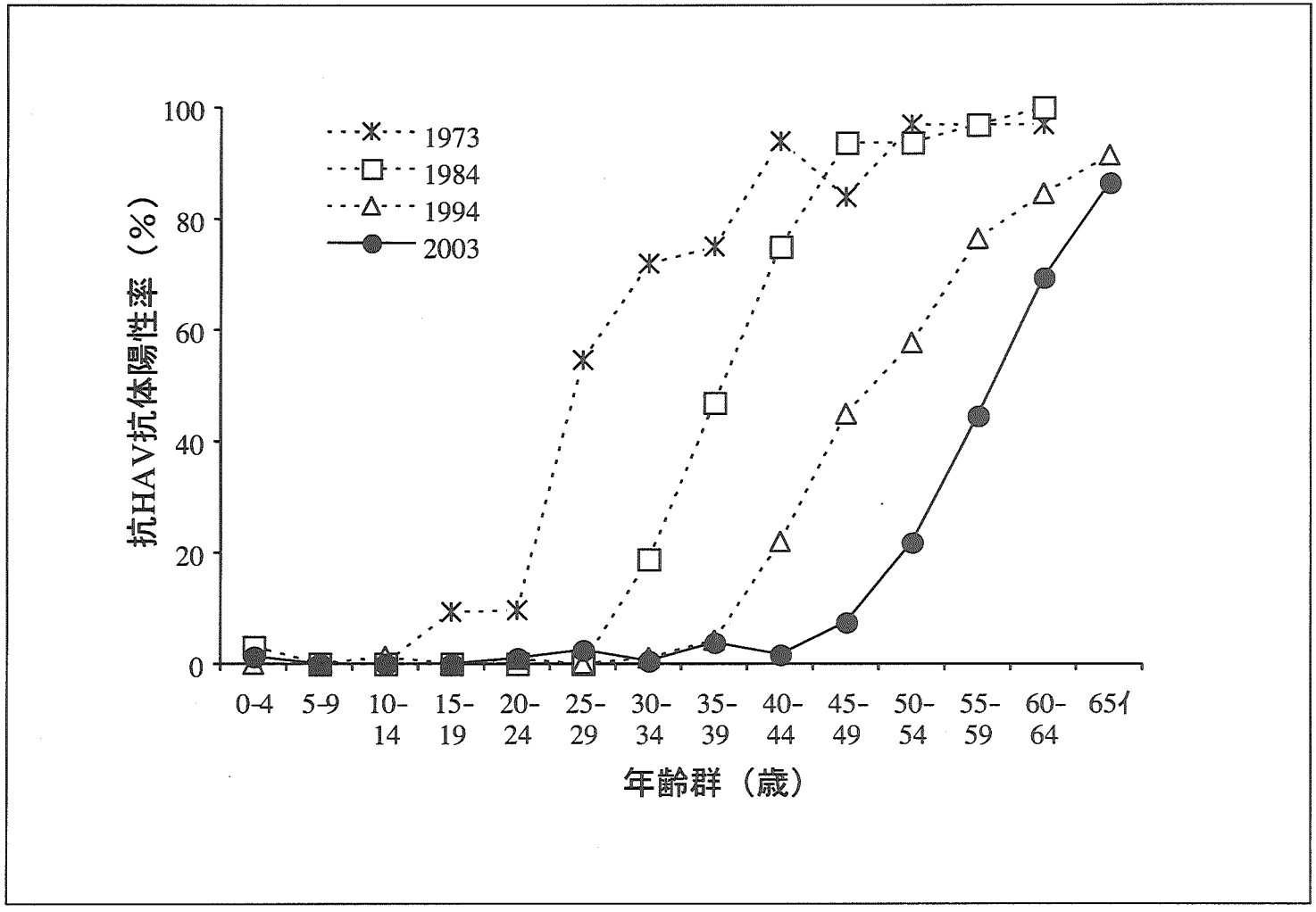

図３．　２００３年の年齢別抗HAV抗体保有状況

厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）
「ウイルス性食中毒の予防に関する研究」
分担研究報告書

沖縄に生息するマングースのE型肝炎ウイルス抗体保有状況

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研究要旨　2002年から2005年まで沖縄で捕獲した199匹のマングースを対象として、血清中の抗HEV IgG, IgM抗体、 およびHEV RNAを調べた。IgG陽性率は22.1%、IgMのそれは0.5%であった。HEV RNAは全て陰性であった。マングースがHEVに暴露されていることが明らかになったが、真のリザーバーであるか否かを結論づけるには、遺伝子の検出と感染実験が必要である。

A．研究目的

E型肝炎の原因ウイルスであるE型肝炎ウイルス（HEV）はプラス一本鎖RNAを遺伝子に持つ小型球形ウイルスである。E型肝炎は先進国においては輸入感染症と思われてきたが、近年、まったく海外渡航歴のないE型肝炎患者が見つかるなど、わが国においても既に土着しているウイルスと考えられている。また、わが国のブタやイノシシは高いHEV抗体保有率を示し、ヒトHEVに遺伝学的に極めて類似するウイルスも分離されている。したがって、これらの動物がHEVのリザーバーであるかもしれない。沖縄島には1910年にハブやネズミの駆逐を目的にジャワマングース(*Herpestes javanicus*)が移入され、現在、広範囲に生息が確認されている。本研究では沖縄島における動物のHEV感染を明らかにする目的で、マングースの抗体およびHEV遺伝子の保有状況を調べた。

B．研究方法

沖縄島に生息する野生のマングース199匹を捕獲し、血清を採取した。HEV組換え中空粒子をマイクロプレートに固相化し、HRP-anti
cat IgGおよびIgMを二次抗体としたELISA法を確立し、マングース血清中の抗体を検出した。抗体の有無はウエスタン法で確認した。また、血清からRNAを抽出し、RT-PCR法によって構造蛋白領域の一部を増幅した。

C．研究結果

2002年から2005年まで沖縄で捕獲した199匹のマングースを対象として、血清中の抗HEV IgG, IgMおよびHEV RNAを調べた。199検体中44検体にIgG抗体を検出した。その陽性率は22.1%であった。1検体だけがIgM陽性であり、その陽性率は0.5%であった。年度別のIgG抗体保有率は2002年が46.2% (12/26)、 2002年が:46.2%(12/26)、2004年が10.1% (7/69)、2005年が23.4% (18/77)で、年度によって抗体保有率が異なっていた。

これは捕獲地域の違いによるものと考えられる。IgGの陽性率はマングースの体重および身長の増加とともに高くなる傾向も見られた。現在までRNA陽性例はまだ見つかっていない。

D．考察

マングースは日本の固有動物種ではない。ハブの駆逐のため1910年、インドから沖縄に、1979年、鹿児島の奄美に導入された。日本にはマングースの天敵がいないため、繁殖スピードは非常に速かったと考えられる。また、日本に生息しているマングースはハブの捕食はほとんどせず、希少な動物と鳥類を食べることから、生態系のバランスを脅かす存在となっている。そのため、現在沖縄、鹿児島ではマングースの駆除が実施されている。沖縄ではHEVの宿主と思われる琉球イノシシが数多く生息している。同じ地域で生息しているマングースがイノシシの糞便とともに環境に排泄されたHEVに暴露されることが十分考えられる。マングースにおける抗体保有率および遺伝子の検出を調べることは、動物間でのHEVの伝播の状況を把握する上で重要である。

本実験からIgG抗体の保有率は22.1%に上ることが示され、マングースがHEVに暴露されていることが明らかになった。野生動物であるマングースの年齢を正確に断定するには難しいが、抗体保有率は体重と身長とともに増加する傾向が見られたことから、抗体保有率は年齢と相関することが示唆された。現時点ではウイルス遺伝子そのものはまだ見つかっておらず、HEVが真にマングースに感染するかどうか不明である。

E．結論

沖縄に生息しているマングースの抗体保有率は22.1%で、HEVに暴露されていることが明らかになった。マングースがHEVのリザーバーであるかどうかは、遺伝子の検出と感染実験が必要である。

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1．特許申請：なし
2．実用新案登録：なし
3．その他：なし

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Multiprefectural Spread of Gastroenteritis Outbreaks Attributable to a Single Genogroup II Norovirus Strain from a Tourist Restaurant in Nagasaki, Japan

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A series of gastroenteritis outbreaks caused by noroviruses (NVs) among tourist groups from several prefectures was associated with eating a lunch prepared by a restaurant in Nagasaki City, Japan, on 18 and 19 November 2003. A retrospective cohort study was performed to estimate the magnitude of the outbreak and identify the source of infection. Epidemiological information was obtained through the local public health centers in the areas where the illness occurred. Stool and vomit specimens and food and environmental samples were analyzed by reverse transcription-PCR with genogroup-specific primers. Positive samples were sequenced and analyzed phylogenetically. Of 1,492 tourists who ate a lunch prepared by the restaurant during the 2-day period, 660 (44.2%) developed illness, with an average incubation time of 31.2 h. Whereas NVs were not detected in any food samples, identical sequences most closely related to the Mexico genotype of genogroup II NV were found in specimens from case patients, restaurant staff, and the kitchen table. Food handlers were concluded to be the source of the outbreak as a result of the contamination of several meals. The series of outbreaks described here exemplifies the role of tourism as a contemporary way to distribute a single infectious agent to multiple and geographically remote areas.

Norovirus, a genus within the family *Caliciviridae*, has emerged as an important cause of food- and waterborne gastroenteritis outbreaks in industrialized countries (6, 8, 20). Noroviruses (NVs) are responsible for 78.5% of all nonbacterial outbreaks of gastroenteritis reported from 1995 to 2000 in Europe (21). They accounted for an estimated 6 to 14, 11 to 18, and 20% of infectious intestinal diseases in England and Wales (3, 7, 28), The Netherlands (4, 17), and Finland (27), respectively. It was reported that 96% of 90 outbreaks of nonbacterial gastroenteritis were caused by NVs (6), and it is estimated that NVs cause 23 million illnesses each year (22) in the United States. In Japan, NVs accounted for 28% of cases of food poisoning from all causes and 99% of cases from purely viral sources (24).

NVs can be classified into five genogroups, genogroups GI to GV; the three genogroups GI (prototype strain, Norwalk virus), GII (prototype strain, Snow Mountain virus), and GIV have been found in humans (1, 23, 29, 31). Reverse transcription-PCR (RT-PCR) has become a favored method for detection and classification of NVs and has extensively been used as a tool in investigations of acute gastroenteritis outbreaks (9, 13, 30, 33). Little has been reported about the genotype distribution of NVs in Japan. The GII Lordsdale genotype (GII/4) has been predominant since 1996, and the GII Mexico genotype (GII/3) suddenly appeared and spread during the 1999-2000 season in Osaka City, Japan (11). In another study, various genotypes of NVs were found in Kyushu, Japan, from 1988 to 1993, and the GII Mexico genotype was dominant in 1989 (26). In Japan, raw oysters are the primary source of transmission in small outbreaks, whereas school lunches and catered meals, banquet halls, and hospitals are most often implicated as the vehicles and settings of transmission in large outbreaks (those involving >50 patients) (10). In terms of the number of patients involved in NV gastroenteritis outbreaks in Japan, the largest one (3,236 schoolchildren) occurred in nine elementary schools in 1989 following consumption of a school lunch prepared by a lunch preparation center in which one food handler had gastroenteritis (15).

In this article we describe the investigation into a series of gastroenteritis outbreaks that occurred among tourists who had a lunch prepared by a single tourist restaurant and that were attributed to a single strain of NV.

MATERIALS AND METHODS

Outbreak description. Multiple outbreaks of acute gastroenteritis occurred among the tourists from several prefectures who visited Nagasaki City, Japan, and who had a lunch prepared by a tourist restaurant (restaurant J) in November 2003. Nagasaki City is located in the western part of the island of Kyushu, has a population of 420,000, and is visited by more than 5 million tourists a year. On 19 November, the Public Health Authority in Nagasaki City initially received two independent calls that students and teachers from schools in different prefectures who had visited Nagasaki City on a school excursion the day before had gastrointestinal symptoms, such as nausea, vomiting, diarrhea, abdominal pain, and fever. It turned out that the members of these tourist groups had lunch at restaurant J or ate box lunches prepared by that restaurant.

Thus, the Public Health Authority immediately suspended the business of restaurant J, as it was the suspected origin of the food-poisoning outbreak. Gastroenteritis cases continued to occur among tourists who had lunch prepared

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TABLE 1. Characteristics, attack rates, and incubation times for the groups that ate at restaurant J on 18 November

Group	Type of group	Time of visit	No. of tourists	No. of patients	Attack rate (%)	Incubation time (h)[a]
A	Junior high school excursion from Kagoshima	Box lunches	32	25	78.1	27.8 ± 7.1
B	Adults from inside Nagasaki Prefecture	11:30	26	2	7.6	33.5 ± 2.8
C	Elementary school excursion from Fukuoka	11:40	103	73	70.9	33.0 ± 8.6
D	Adults from Osaka	12:30	17	5	29.4	30.1 ± 10.6
E1	High school excursion from Aichi	12:30	415	322	77.6	29.8 ± 10.3
Total			593	427	72.0	30.1 ± 10.0

[a] Values are means ± standard deviations.

by restaurant J on 19 November. Restaurant J was open for tourist groups only on a subscription basis and had a kitchen staff of 10, including 2 cooks, at the time of the event. Single parties of less than 30 tourists each visited restaurant J each day between 15 and 17 November. However, a total of 11 groups ate food from restaurant J on 18 and 19 November; 593 tourists among 5 groups (groups A to E1) ate food from the restaurant on 18 November, and 931 tourists among 7 groups (groups A, E2, and F to J) ate food from the restaurant on 19 November (Tables 1 and 2).

Epidemiological investigation. A retrospective cohort study of the 11 groups that ate food from restaurant J on 18 and 19 November was conducted. Since the case patients became ill at home or during their trip after they left Nagasaki City, information was obtained through the local public health centers in the administrative regions where the case patients affected by gastroenteritis lived. The questionnaires, standardized by the Ministry of Health, Labour and Welfare, were used to obtain information about the sex and age of each of the patients, the time of onset and nature of their symptoms, and what foods they ate.

A case was defined as the development of at least two of the following symptoms in any tourist who had eaten food from restaurant J on 18 and 19 November: nausea, vomiting, diarrhea, abdominal pain, and fever.

The restaurant employees were interviewed in detail. We investigated the hotels and other restaurants in Nagasaki City that the 11 groups used during their trips. We also interviewed other tourist groups that visited Nagasaki City during the same period but that did not consume food from restaurant J. Information on the secondary cases was gathered through the local public health centers.

Environmental investigation. The facility was inspected by the Food Hygiene Section of the Nagasaki City Health Department on 20 and 21 November. The storage conditions of the meals and bulk food items were investigated, and several food samples were taken. A total of 29 smears of environmental samples were also taken from the restaurant, including the kitchen and the washroom. Stool specimens from all kitchen staff were submitted on 21 and 22 November.

Microbiological investigation. The vomit and stool specimens from the case patients were cultured for bacterial enteropathogens, including *Salmonella*; *Shigella*; enteropathogenic *Escherichia coli*, including *E. coli* O157; *Campylobacter*; *Yersinia*; *Vibrio*; *Aeromonas*; *Plesiomonas*; *Staphylococcus aureus*; *Clostridium perfringens*; and *Bacillus cereus*. Approved standard laboratory methods were used for all bacteriological investigations.

RNA extraction, RT-PCR, and sequencing. Samples and specimens were examined for NVs by RT-PCR, as described elsewhere (24, 33). Genogroup-specific primers were used to amplify the partial capsid region of NVs by RT-PCR (16, 24), as follows: primers COG1F and G1-SKR and primers COG2F and G2-SKR for amplification of the GI and GII NVs, respectively. For some samples, a nested PCR was performed with primers G1-SKF and G1-SKR (GI) and with primers G2-SKF and G2-SKR (GII). We also quantified the NV capsid genes for some PCR-positive samples by using a real-time PCR, as described previously (13, 24). The detection limits were 10^1 and 10^2 copies for the food and environmental samples and the clinical specimens, respectively (data not shown).

The capsid sequences were aligned, and the nucleotide sequence identities were analyzed with GENETYX-MAC software (version 11.0). The nucleotide sequences were compared with those of reference strains of NVs obtained from GenBank for the phylogenetic analysis, as described previously (14).

Statistical analysis. Data are presented as means (standard deviations and ranges) or as counts or proportions. Student's t test was used to compare the means between the two groups. The chi-square test was used to assess the statistical significance of the associations among variables. We calculated odds ratios (ORs) using Woolf's procedure and multivariate ORs using multiple logistic regression analysis (SAS, version 8.2) for each group and Mantel-Haenszel ORs for all subjects together, with 95% confidence intervals (CIs), to assess whether there was any association between illness and an individual meal, food, or food item. A P value less than 0.05 was considered significant.

Nucleotide sequence accession number. The NV capsid sequence data have been submitted to GenBank and assigned accession number AY590117.

RESULTS

Epidemiological investigation. All 10 tourist groups in which gastroenteritis cases occurred had eaten lunch at restaurant J or ate box lunches prepared by this restaurant. By contrast, there were no reports of illness among 44 tourist groups (2,371 persons) who visited Nagasaki City during the same period but who did not dine at restaurant J ($P < 0.001$). No hotels or restaurants, other than restaurant J, where the 10 groups stayed or visited reported the occurrence of gastroenteritis. Consequently, restaurant J was concluded to be the causative facility of the outbreak.

Tables 1 and 2 show the times and the dates when the

TABLE 2. Characteristics, attack rates, and incubation times for the groups that ate at restaurant J on 19 November

Group	Type of group	Time of visit	No. of tourists	No. of patients	Attack rate (%)	Incubation time (h)[b]
F	High school excursion from Hokkaido	Box lunches	163	97	59.5	34.2 ± 10.4
G	Elementary school excursion from Kumamoto	11:00	145	37	25.5	28.3 ± 12.7
E2	High school excursion from Aichi	11:45	294	63	21.4	39.3 ± 16.4
H	Elementary school excursion from Kumamoto	12:10	169	35	20.7	24.5 ± 15.0
I	Adults from Gunma	12:10	15	0	0.0	
A	Junior high school excursion from Kagoshima	12:40	32	25[a]	78.1[a]	27.8 ± 7.1[a]
J	Junior high school excursion from Kagoshima	12:50	113	1	0.9	25.8
Total			931	233	25.9	33.1 ± 14.2

[a] The case patients were thought to be infected on the first day because of the incubation period.
[b] Values are means ± standard deviations.

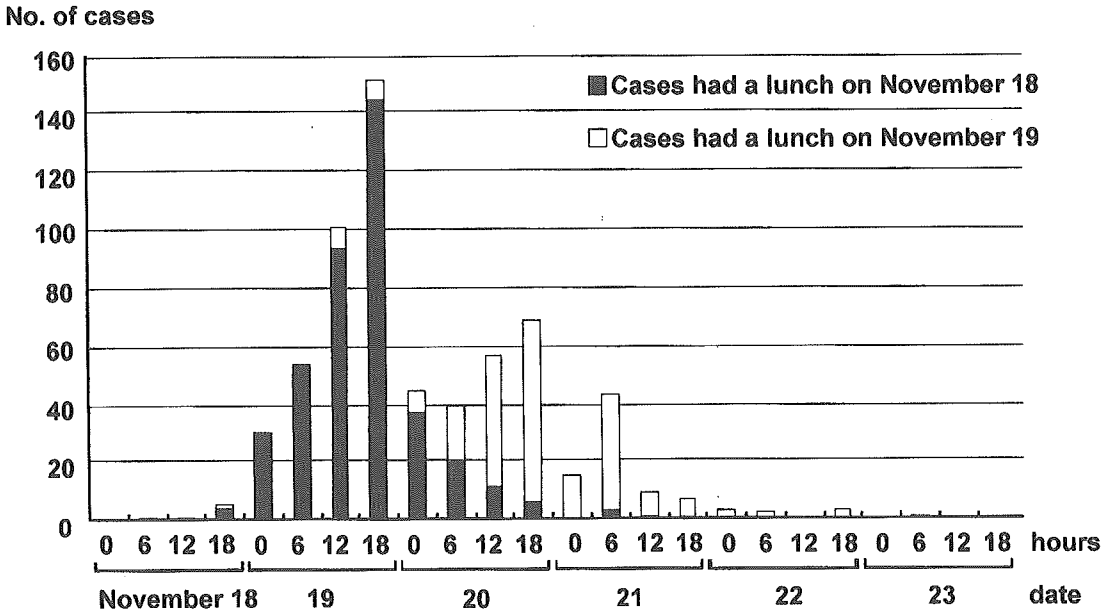

FIG. 1. Epidemic curve of cases, by hours and dates of onset of symptoms. The *x* axis presents the times (in hours) and the days when the onset of symptoms occurred.

tourists visited Restaurant J, the type of tour, the numbers of tourists and cases, the attack rates, and the incubation times for each group. Group A consumed meals from restaurant J on both 18 and 19 November. Groups E1 and E2 belonged to the same school and visited restaurant J on 18 and 19 November, respectively. The questionnaires were received from 97.3% of the tourists (35.3 to 100% for each group). Most groups responded very well (96.1 to 100%), whereas group D, which consisted of adult individuals only, responded poorly (35.3%).

Of the 1,492 tourists who used restaurant J, 660 developed illnesses that met the case definition. Thus, the overall attack rate was 44.2%. The mean age was 17.0 ± 8.4 years (age range, 11 to 74 years); and 90.6% of the cases occurred among students in elementary, junior high, and high schools (age range, 11 to 18 years). There was no sex-related difference in the attack rates, which were 46.8% for males and 44.7% for females ($P = 0.64$). The attack rates were invariably greater than 70% for the students who had lunch at restaurant J on 18 November, while they gradually decreased for those who had lunch there on the next day. There was a significant difference ($P < 0.001$) in the attack rates between the groups that ate lunch on the first day (72.0%) and the next days (25.9%) of the outbreak. The attack rate was low in groups B and D, and there were no illness in group I; although the amounts and types of foods consumed did not differ, all these groups were commonly adult tourist parties. The symptoms most commonly reported by case patients were nausea (87.0%), vomiting (71.8%; 4.0 times a day, on average), abdominal pain (69.5%), fever (68.6%), and diarrhea (54.4%; 3.1 times a day).

The epidemic curve shows two peaks (Fig. 1), but each peak represents a cluster of cases among those who ate food from the restaurant on either 18 or 19 November and has a pattern characteristic of a single-exposure, common-vehicle outbreak. The mean incubation time was 31.2 ± 11.7 h, and there was no difference in the incubation times between the tourists who consumed food from restaurant J on the first day (30.1 ± 10.1 h) and those who consumed food from the restaurant on the next day (33.1 ± 14.2 h) ($P = 0.32$).

Groups A and F had box lunches prepared by restaurant J and commercially available tea in a plastic bottle and consumed the box lunches on a ferry and a train, respectively. The same food items were assorted in the box lunches for these two groups. All other groups had lunch at restaurant J and had cold tea prepared by the restaurant. Although the combination of foods was not always identical, most foods were common in the lunches served to each group. When analysis was performed for each group separately, illness was statistically significantly associated with a specific food in three groups: Sara-Udon (thin fried rice noodles with mixed vegetables and seafood) in group C (OR, 3.1; 95% CI, 1.1 to 8.7; $P = 0.03$), deep-fried spring roll in group E1 (OR, 2.3; 95% CI, 1.1 to 4.7; $P = 0.02$), and boiled broccoli in group F (OR, 2.4; 95% CI, 1.2 to 4.6; $P = 0.01$). When analysis was performed for all subjects stratified together by group and day, deep-fried spring roll (Mantel-Haenszel OR, 2.06; 95% CI, 1.39 to 3.05; $P = 0.0004$), boiled broccoli (Mantel-Haenszel OR, 2.41; 95% CI, 1.29 to 4.51; $P = 0.009$), and raw lettuce (Mantel-Haenszel OR, 2.12; 95% CI, 1.13 to 3.95; $P = 0.03$) were significantly associated with illness. It may deserve to be mentioned that the four food items described above, the Sara-Udon, deep-fried spring roll, boiled broccoli, and raw lettuce, were handled with bare hands after cooking or washing. However, none of the groups were served all four of these items together. When deep-fried spring roll, boiled broccoli, and raw lettuce were included in the same model simultaneously, only boiled broccoli was significantly associated with illness (multivariate OR, 2.0; 95% CI, 1.0 to 3.9; $P = 0.05$) in groups A and F, to which all three of these

food items were served. However, none of these items that was significantly associated with illness was common to all groups.

There were two reports on the occurrence of secondary cases, besides the tourists: (i) NVs were detected in 2 sick employees of the hotel where group E stayed on the trip after visiting Nagasaki City, and (ii) 21 family members of 16 case patients in group C became sick.

Environmental investigation. On 14 November, the chief cook who was in charge of food hygiene at the kitchen had quit his job. This loss of staff, together with an extraordinary number of guests, made the business in the kitchen of the restaurant hectic during the 2-day period. One of the cooks felt general fatigue from 16 November and took an over-the-counter cold medicine on 19 November, although he allegedly had no gastrointestinal symptoms. No other restaurant staff allegedly had any illness during or immediately before the event. None of the employees reported that they had eaten raw shellfish, such as oysters, during the several days prior to the outbreak, and no family members of the employees were sick. All kitchen staff had eaten at least one meal at restaurant J on 18 and/or 19 November.

Restaurant J had only one washroom, which was located adjacent to the kitchen and which was used by both employees and tourists. Since there was no sink for hand-washing in the kitchen, the cooks washed their hands in the sink used to wash vegetables and kitchenware and wiped their hands on their aprons. The cooks and the other food handlers mostly handled the food items with their bare hands. Containers were commonly used for the food items before and after cooking. The same chopping board was used for different food items. The lettuce for the box lunches was washed with bare hands and soaked in water overnight, as was the boiled broccoli. The cold tea was prepared in a big bucket with hot water and then cooled with cubes of ice made in the ice machine in the kitchen.

In addition to the 29 environmental samples, a total of 58 meals served between 15 and 19 November were stored for the investigation and 9 bulk food items, such as frozen seafood, including bivalves similar to clams (*Paphia vernicosa*), had been kept during the inspection and were available for the investigation.

Microbiological investigation. Stool specimens (from 77 case patients) and vomit specimens (from 54 case patients) were obtained from a total of 124 case patients. Although *S. aureus* enterotoxins were detected in two vomit specimens from students in group E, the toxins from the two case patients were different: enterotoxin A and enterotoxin B, respectively. *Aeromonas hydrophila* was detected in a stool specimen from a case patient in group F. No enteropathogenic bacteria were detected in the other case patients, stool specimens from the kitchen staff, or the environmental samples from restaurant J.

RT-PCR and sequencing. Amplification by RT-PCR with genogroup-specific primers demonstrated the presence of 387-bp bands corresponding to GII NV (Fig. 2). GII NVs were detected in 87 of 124 case patients (70.2%; 44 of 54 vomit specimens [81.5%] and 48 of 77 stool specimens [62.3%]). No food samples were positive for NV, even after the nested PCR. Of the 29 environmental samples tested, only 1 was positive for GII NVs by the nested PCR (product size, 344 bp) (data not shown), and this sample was taken from the table where Sara-

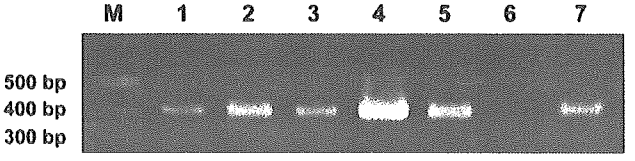

FIG. 2. Detection of NV capsid genes from specimens and samples by RT-PCR with genogroup-specific primers. The PCR products were electrophoresed on a 1.5% agarose gel. Lane M, marker (100-bp ladder; New England BioLabs Inc., Beverly, Mass.); lane 1, fecal specimen from the cook with general fatigue; lane 2, fecal specimen from another member of the kitchen staff (server); lanes 3 to 5, fecal specimens from representative case patients; lane 6, negative control (free of viral DNA); lane 7, positive control for genogroup II (strain Arg320; GenBank accession number AF190817). The GII NVs capsid gene (387 bp) was amplified and detected in the fecal specimens (lanes 1 to 5).

Udon was dished up. GII NVs were also detected in the stool specimens from 5 of 10 kitchen staff, including 2 cooks and 3 servers.

Real-time PCR quantification of the NVs revealed 61.5 copies/cm^2 in the table sample and 3.7×10^8 to 9.4×10^9 copies/g in the stool specimens from the kitchen staff. The capsid sequence analysis revealed that the NVs in all samples from the case patients, the kitchen staff, and the environmental sample had identical sequences (GenBank accession number AY590117). The genotype is most closely related to the well-characterized genotype Mexico/89/MX (GenBank accession number U22498), with 94.9% identity at the nucleotide sequence level (Fig. 3). The sequence in GenBank most closely related to the sequence that we obtained was Oberhausen455/01/DE (GenBank accession number AF425768), with which our sequence had 98.9% identity at the nucleotide level and which was originally from an outbreak in Germany.

DISCUSSION

To our knowledge, this is the largest food-borne gastroenteritis outbreak in terms of the distribution from a single causative facility into diverse geographic locations across the country, and the existence of an outbreak was unambiguously shown by linking classical and molecular epidemiological measures to a single GII NV strain of the Mexico genotype. Although recent papers have shown that new GII/4 NVs emerged in Europe (18) and on cruise ships in the Unites States (32), the causative NV in our study was classified as a different subtype, subtype GII/3. The outbreak described here is thought to be unique in that several tourist groups from across Japan were affected with gastroenteritis by exposure to NVs from a specific restaurant during a defined period of time and became ill at home or on the continuation of their trips; consequently, the specific virus has since spread into multiple prefectures. Such spread of a single infectious agent by travelers who play the role of disease transmission vehicle should be cautionary, as the outbreak is further proof of one of the contemporary modes of transmission of infectious diseases. Actually, Beller et al. (2) reported on a waterborne outbreak of illness caused by NVs in tourists traveling by bus between the United States and Canada. Furthermore, Noel et al. (25) reported that NV outbreaks due to a single virus occurred in seven countries on five continents during the 1995-1996 sea-

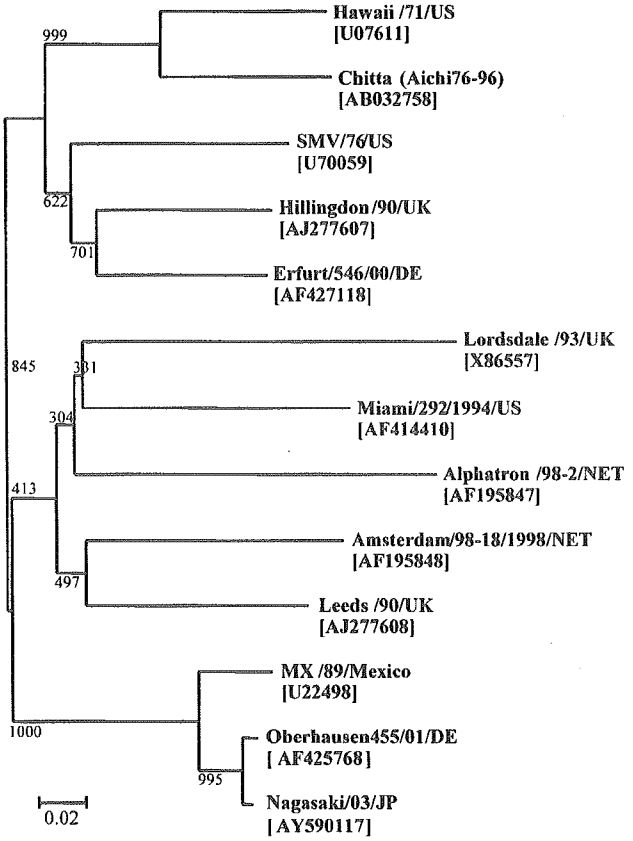

FIG. 3. Phylogenetic tree constructed on the basis of the sequences of a part of the capsid gene of GII NVs from the present outbreak and known strains from the GenBank database. GenBank accession numbers for the strains are indicated in the parenthesis. The causative viral strain of the present outbreak is shown as Nagasaki/03/JP. The numbers at each branch indicate bootstrap values for the clusters supported by that branch.

son, suggesting that the circulation of the strains might involve patterns of transmission not previously considered.

Food-borne vehicles of NVs are typically contaminated bivalve shellfish, such as oysters, items contaminated by infected food handlers, or vegetables or fruit contaminated by irrigation or washing (20). In restaurant J, frozen imported bivalve shellfish was initially suspected as the cause of infection, but no NV was detected in either the shellfish or other food samples. In outbreaks originating from infected food handlers, specific food is not always identified as the main source of the infection (5, 12, 15). Lopman et al. (19) have recently reported that specific vehicles were implicated in 39.1% of NV food-borne outbreaks and that multiple food vehicles contributed to some outbreaks. In the present outbreak, it is still unknown whether a sick cook was first infected with NV and subsequently other kitchen staff and tourists were infected or whether the kitchen staff was infected simultaneously with tourists by unknown transmission routes. However, we believe that several foods were contaminated by employees working at restaurant J. This is supported by the facts that (i) identical NVs were detected from the kitchen staff, the kitchen environment, and case patients; (ii) no NVs were found in meal or food samples; (iii) no common foods were suspected as the main source of infection; (iv) there were no differences in the attack rates between groups of tourists who ate box lunches prepared by the restaurant and those who ate at the restaurant, even though there was a great difference in the combinations of foods consumed; (v) the kitchen staff mostly handled food items with bare hands; and (vi) the kitchen staff used poor food-handling hygiene.

Although we failed to obtain a sample of ice tea prepared by the restaurant, the attack rate among the students who consumed commercially available bottled tea did not differ at all from that among those who consumed ice tea prepared by the restaurant, suggesting that waterborne transmission was much less likely. Indirect contamination in the washroom was also less likely because NV was not detected in the washroom and the illness occurred in the tourists who did not visit the restaurant. Unfortunately, we failed to obtain a sample of water in which the lettuce and broccoli were soaked overnight. The attack rates were significantly lower in the tourists who ate food from the restaurant on 19 November than those who ate food from the restaurant on 18 November ($P < 0.001$), and the rates dropped steeply on 19 November, suggesting that the foods were substantially more contaminated on 18 November (Tables 1 and 2). The fact that the attack rates for groups E1 and E2 (77.6 and 21.4%, respectively), which had the same background, showed a significant difference ($P < 0.001$) supports this hypothesis (Tables 1 and 2).

Although the highest incidence of NV infections is in children under 5 years, NV infections can occur at any age (20). In the outbreak reported here, all tourists ate a similar combination of foods at the restaurant, while the attack rates for adult tourist groups were much lower than those for student tourist groups. This suggests that NV gastroenteritis may tend to cause more severe illness in children and adolescents than in adults. This is consistent with the findings of a proportion analysis study conducted in The Netherlands (4), which showed that individuals in the age group of 18 to 64 years demonstrated a lower infection rate than individuals in younger and older age groups. Although the average incubation time in the present outbreak was thought to be typical for primary NV gastroenteritis, it is possible that some cases with apparently longer incubation periods were probably due to secondary person-to-person transmission, since most tourist groups continued their tours after they left Nagasaki City.

The sudden emergence and spread of a single strain raise important public health implications about the mode of transmission that permitted the rapid radiation of a single virus (6). It is generally believed that the movement of people from one place to another, whether it is through tourism or other means, may have profound effects on the dissemination of NVs into different populations, but there is not much evidence that directly supports such a hypothesis. In this regard, this study provides a unique opportunity to gain insight into the question of how various genotypes of NVs emerge, cocirculate, and disappear in different geographic locations. It is important and interesting to use modern molecular biology-based techniques to keep track of where this NV outbreak strain will spread and if it will cause outbreaks in Japan or elsewhere in the world. For this purpose, enhanced vigilance that includes the pursuit and characterization of secondary cases that follow outbreak

cases is continuously needed. It is also essential that food samplers not work when they are ill and that good handwashing facilities be provided in all restaurants.

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Genetic diversity of sapovirus in fecal specimens from infants and children with acute gastroenteritis in Pakistan

Brief Report

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Summary. A total of 517 fecal specimens collected from infants and children with acute gastroenteritis in Karachi city, Pakistan during 1990–1994 were examined for the presence of sapovirus by RT-PCR and sequence analysis methods. Sapovirus was identified in 17 of 517 (3.2%) specimens. Sapovirus was further clustered into three distinct genogroups (I, II and IV) and these presented 70.6%, 23.5% and 5.9%, respectively. Our results clearly indicated that sapovirus could be classified into 7 GI and 4 GII genotypes. It was noteworthy to point out that sapovirus detected among Pakistani infants and children with acute gastroenteritis demonstrated the great genetic diversity and presented novel sapovirus genotypes.

*

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis consistently ranks as one of the top six causes of all deaths. Apart from rotavirus as the major etiologic agent of gastroenteritis in children and young animals, sapovirus is considered to be a significant global enteropathogen [6, 7].

Sapovirus was first named after its discovery in an outbreak of acute gastroenteritis in a home for infants in Sapporo, Japan in October 1977 [1]. They have a typical "Star of David" configuration by EM and are antigenically identical to each other by immune EM [2]. Sapovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. Sapovirus genome contains

two ORFs (ORF1 and 2); ORF 1 encodes the non-structural as well as the capsid proteins. Based on the sequence analysis of the capsid gene, sapovirus is divided into three genogroups (I, II and IV) and currently genogroup V as known to infect human [3, 9, 15]. Moreover, porcine enteric calicivirus (PEC) is reported to be a member of genogroup III [4].

Etiologic studies of acute gastroenteritis in infants conducted in Japan with sensitive reverse transcription–PCR methods showed that sapovirus was one of the most causes of outbreaks of viral gastroenteritis among infants in Sapporo [1, 2]. This virus also is associated with sporadic outbreaks of gastroenteritis worldwide and is recognized just as important as rotavirus infecting children less than 2 years of age in Finland [10, 11]. Recently, seroepidemiologic studies have indicated a worldwide distribution of sapovirus [6, 7]. The aged-related prevalence of antibody against this virus also has shown that infections commonly occur in children less than 5 years old. The pattern of acquisition of the antibody is similar to that of other common virus infection [14].

The objectives of this study were: to analyze the epidemiology of sapovirus among Pakistani pediatric population with acute gastroenteritis, to characterize the detected sapovirus according to genogroup and genotype, and to describe the genetic diversity of sapovirus.

Five hundreds and seventeen fecal specimens were collected from infants and children with acute gastroenteritis in the Civil Karachi Hospital, Dow Medical College, Karachi city, Pakistan during the period of January 1990 to December 1994. All fecal specimens were determined previously to be negative for rotavirus and adenovirus [8]. These specimens were diluted with Eagle's minimum essential medium to 10% suspensions, and clarified by centrifugation at 5000 g for 20 min. The supernatants were collected and stored at −30 °C until use for the detection of sapovirus. The viral genome was extracted from 140 µl of a 10% fecal suspension using a spin column technique according to the instructions in the QIAamp® Viral RNA Mini Kit Handbook, Germany. Sapovirus was detected by RT-PCR analysis of extracted viral RNA with specific primers previously published [19]. Briefly, a pair of published primers (SLV5317 and SLV5749) for amplifying capsid region of sapovirus was used. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C [19]. PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 5 mg/ml) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography. The nucleotide sequences of PCR products (DNA) positive for sapovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using E-CLUSTAL W (Version 1.6). Reference strains and accession numbers used in this study are as follows: Southampton (L07418), PEC (AF182760), Bristol/89 (AJ249939), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruise ship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Stockholm/97 (AF194182), Mex14917/00 (AF435813), Houston/90 (U95644), Parkville/94 (U73124),

Houston/86 (U95643), Sapporo/82 (U65427), Manchester/93 (X86560), Plymouth/92 (X86559), Lyon/30388/98 (AJ251991), Southampton (L07418).

Out of 517 fecal specimens collected from infants and children with acute gastroenteritis in Karachi city, Pakistan, 168 were collected in 1990; 86 in 1991; 76 in 1992; 99 in 1993; and 88 in 1994. For the pediatric population, the lowest age was under 1 month, the highest was 5 years, and the average age was 1 year (12 months). Moreover, the number of male (60.9%) was higher than that of female (39.1%). All fecal specimens were tested for the presence of sapovirus by RT-PCR. The results shown in Table 1 revealed that sapovirus was detected in 17 out of 517 (3.2%) specimens tested. Sapovirus was identified in each of the five years. The highest incidence fell into 1991 (29.4%, 5 of 17) and the lowest in 1993 (5.9%, 1 of 17). The age at sapovirus infection ranged from 5 months to 3 years. The viral infectious rate was highest in the 6–11 months old group (41.2%) and lowest in children over 35 months or less than 6 months of age (5.9%). Moreover, the number of male infected sapovirus with acute gastroenteritis (58.8%) was higher than that of female.

A total of 17 sapovirus amino acid sequences were analyzed by phylogenetics and grouped using the recent sapovirus capsid region classification scheme of Farkas et al. (2004). Majority of the sapovirus sequences clustered into two distinct genogroupes I and II (GI, GII). Interestingly, one of the sequenced specimens positive for sapovirus, Karachi/730/1992, turned out to belong to a cluster called

Table 1. Characteristics of seventeen sapovirus infections among infants and children with acute gastroenteritis in Karachi city, Pakistan during 1990 and 1994

No.	Patient	Year	Month	Sex	Age	Isolate	Genogroup	Genotype	Accession No.
1	877	1990	August	M	3 y	Karachi/877/1990	GI	5*	AB181133
2	878	1990	August	F	5 m	Karachi/878/1990	GI	6*	AB181228
3	953	1990	September	M	1 y	Karachi/953/1990	GII	1	AB181131
4	1017	1990	October	F	2 y	Karachi/1017/1990	GI	5*	AB181227
5	824	1991	January	M	1 y	Karachi/824/1991	GII	1	AB181130
6	842	1991	May	F	8 m	Karachi/842/1991	GI	1	AB181232
7	872	1991	July	F	6 m	Karachi/872/1991	GI	4*	AB181231
8	934	1991	October	M	9 m	Karachi/934/1991	GI	1	AB181247
9	937	1991	October	M	2 y	Karachi/937/1991	GI	6*	AB181229
10	874	1992	March	M	2 y	Karachi/874/1992	GII	4*	AB181129
11	730	1992	August	M	10 m	Karachi/730/1992	GIV#	N/A	AB126249
12	1021	1992	August	F	9 m	Karachi/1021/1992	GI	4*	AB181230
13	1026	1992	September	F	9 m	Karachi/1026/1992	GI	5*	AB181134
14	876	1993	September	F	1 y	Karachi/876/1993	GI	7*	AB181132
15	928	1994	March	M	1 y	Karachi/928/1994	GII	4*	AB181128
16	938	1994	April	M	9 m	Karachi/938/1994	GI	1	AB181248
17	997	1994	July	M	1 y	Karachi/997/1994	GI	1	AB181233

Note. *No.*, Number; *M*, Male; *F*, Female; *y*, Year; *m*, Month; #Rare genogroup
*New genotype; *N/A*, Not available

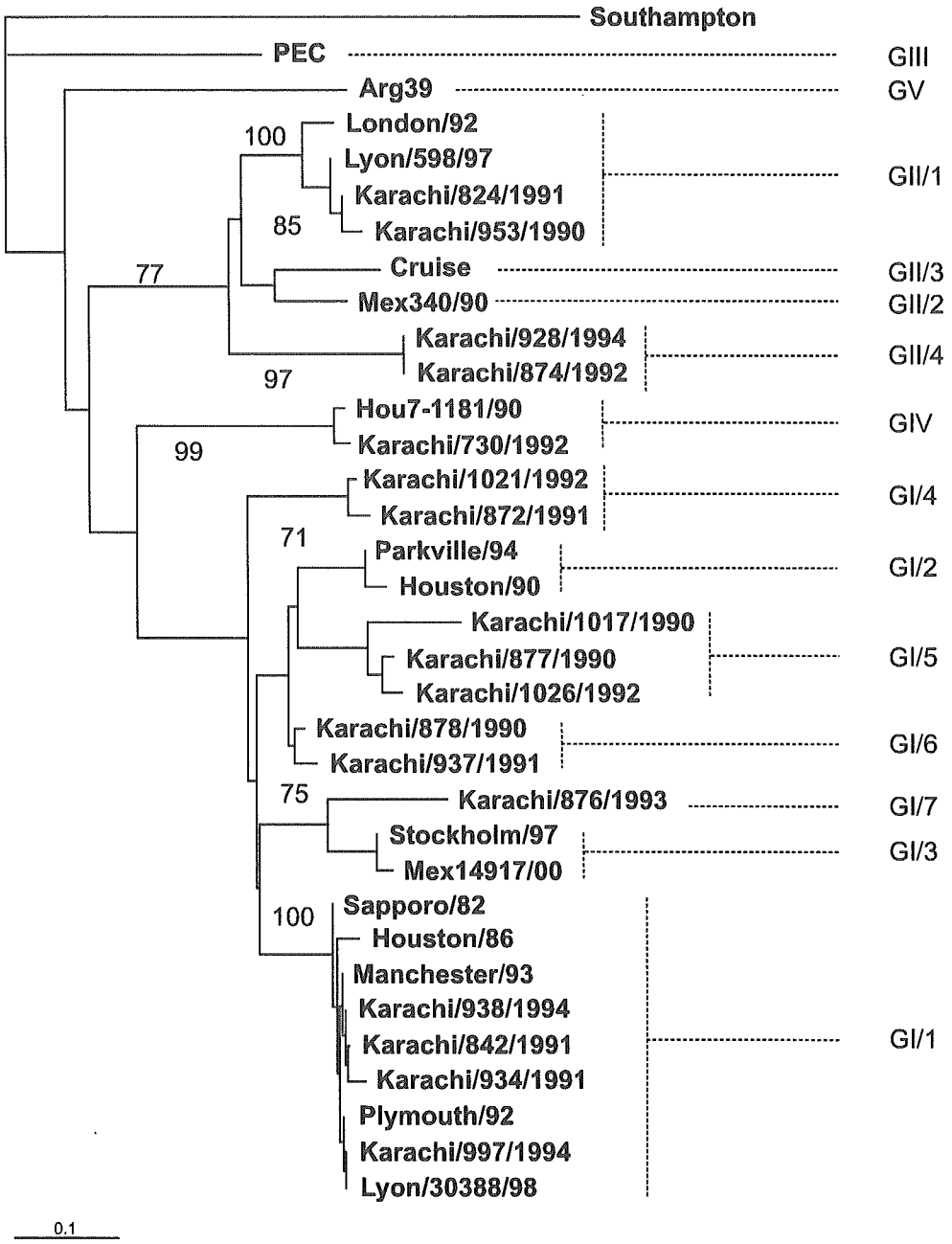

Fig. 1. Phylogentic tree of amino acid sequences of 17 isolates of sapovirus based on the recent sapovirus capsid region classification scheme of Farkas et al. [3]. The tree was constructed from partial amino acid sequences of the capsid region of the seventeen sapoviruses detected in Karachi city, Pakistan during the period of 5 years (1990 through 1994). The numbers in the branches indicate the bootstrap values. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Southampton was used as an out-group strain for phylogenetic analysis

a genogroup IV (known as the Hou7-1181/90 virus). The nucleotide identity between Karachi/730/1992 and Hou7-1181/90 was 96%, and the amino acid identity was 97%.

Most of the sapovirus sequences (70.6%, 12 of 17) belonged to sapovirus GI (known as the Manchester virus cluster). Our results indicated that sapovirus group I was a dominant genogroup. The sapovirus GI sequences clustered into one distinct GI genotype, GI/1 (typified by the Sapporo/82 virus cluster) (33.3%, 4 of 12). In addition we also identified eight isolates (3 in 1990, 2 in 1991, 2 in 1992 and 1 in 1993), forming four novel sapovirus GI genotypes tentatively called GI/4, GI/5, GI/6 and GI/7, respectively (Fig. 1). These sapoviruses had a low identity on the amino acid with other reference strains in the same genogroup ranged from 65% to 86%.

Our findings showed that two (50%) of the sapovirus GII sequences were classified into GII/1 (known as the Lyon/598/97 virus). Interestingly, two isolates named Karachi/928/1994 and Karachi/874/1992 in the present study did not belong to any the genetic clusters and presented a novel sapovirus GII genotype tentatively called GII/4. The sequences with the closest matches to these isolates were from strain isolated in France (Lyon/598/97), showing only 73% amino acid identity.

Sapovirus is one of the leading causes of infantile viral gastroenteritis and also is associated with sporadic outbreaks of gastroenteritis worldwide. In this study, sapovirus was detected in 3.2% fecal specimens tested. These findings suggested that acute gastroenteritis in infants and children in Karachi, Pakistan about 3.2% might be due to sapovirus and 96.8% caused by other etiologic agents. Among all children with acute gastroenteritis due to sapovirus, 94.1% were aged less than 36 months. This result was consistent with previously published reports on epidemiology of sapovirus worldwide in which the prevalence was shown to be 0.3–9.3% [5, 9, 16–18]. It also confirmed sapovirus as one of the enteropathogens responsible for viral gastroenteritis among infants and children worldwide. Furthermore, the highest incidence was the 6–11 month old group, the lowest fell into the infants aged less than 6 months, and the rate of incidence decreased with increasing age over 1 year. Quite possibly, children aged from 6 to 11 months might lack antibody protection to sapovirus, whereas by the time children have reached 1 year old they have begun to acquire viral immunity.

The climate in Karachi city, Pakistan is distinctively seasonal. The summer lasts from April to July, and the hot temperature may reach over 37 °C. The cold season characterized by less rain begins in August and ends in March. The coldest month is January when the temperature may dip as low as 5 °C. In the present study, almost sapoviruses were found in August, September and October. Our results were in line with previous reports of other investigators that sapovirus was observed to prevail in the cold winter months [9, 12, 13, 18].

Up to date, numerous molecular epidemiological studies have revealed a global distribution of sapovirus. However, the genetic analysis on sapovirus in Pakistani children with acute gastroenteritis is not available. The results in this study showed