plastic disposable tube sterilized by electron beam. After incubation at 35°C for 24 h, the development of a blue-green colour in an initially light yellow-coloured solution demonstrates the presence of coliforms and fluorescence at 366 nm in the same tube demonstrates the presence of *E. coli*. Since a DST method has been used as a Japanese standard method since 1992 (Japan Water Works Association [JWWA] 2001), it was decided to use this DST method to confirm the validity of EC-Blue-10 for rapidly and simultaneously detecting coliforms and *E. coli* in temperate humid climate zone waters sampled in Japan.

Materials and methods

EC-Blue-10

EC-Blue-10 consists of a granulated medium in a special plastic bottle sterilized by electron beam. The medium was developed primarily for the rapid growth of *Enterobacteriaceae* (Kodaka *et al.* 1995) and contains the following ingredients g l^{-1}: Trypticase peptone (Becton, Dickinson and Company, Sparks, MD, USA) 5·0, NaCl 5·0, K_2HPO_4 4·0, KH_2PO_4 1·0, KNO_3 1·0, sodium pyruvate 1·0, sodium dodecyl sulphate (SDS) 0·1, MUG 0·1, X-Gal 0·1, isopropyl-β-D-thiogalactopyranoside 0·1 and pH 7·1 ± 0·2.

Effect of media for the detection of coliform bacteria from chlorinated water sample

Escherichia coli American Type Culture Collection (ATCC, VA, USA) 11775, *Citrobacter freundii* ATCC 8090, *Enterobacter cloacae* ATCC 13047 and *Klebsiella pneumoniae* ATCC 13883 were used in this study. Each coliform bacterium was suspended in sterilized phosphate buffer saline (PBS) solution prepared to make 20 000 CFU 400 ml^{-1} in 500 ml Erlenmeyer flask. The bacteria suspension was kept in the water bath at 20°C until mixing with chlorine solution. The chlorine solution was prepared to a target concentration of 0·3 mg l^{-1} (0·3 ppm) in 400 ml sterilized PBS solution in 500 ml Erlenmeyer flask. The chlorine solution was kept in the water bath at 20°C until mixing with the bacterial suspension. The 400 ml bacterial suspension and 400 ml chlorine solution were mixed promptly in 1000 ml sterilized brown Erlenmeyer flask with stopper. This mixed solution was stirred by stirring bar and 100 ml mixed solution was taken at 15, 30, 60, 120 and 300 s after adding sodium hypochlorite (NaOCl) into 300 ml sterilized Erlenmeyer flask containing 0·5 ml of 1 mol l^{-1} of sodium thiosulfate. Each 50 ml sample was diluted with 450 ml sterilized PBS solution. About 10 ml of diluted sample was inoculated into 10 tubes of EC-Blue-10, Colilert-MPN, Lactose Broth with bromothymol blue (LB: Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) and Brilliant Green Lactose Bile broth (BGLB: Nissui Pharma.). These cultures were incubated at 36 ± 1°C for 48 h. During the 48-h incubation, the positive reaction was observed at 20, 24, 28 and 48 h. This experiment was done three times for each strain. The comparison for detection of each coliform from chlorinated water samples was carried out to sum up the positive results from the data of three experiments.

Influence of heterotrophic bacteria for coliforms and *E. coli* detection

Each coliform bacterium (*E. coli* ATCC 11775, *Cit. freundii* ATCC 8090, *Ent. cloacae* ATCC 13047 and *Kl. pneumoniae* ATCC 13883) was suspended in 250 ml sterilized PBS solution to make 20 CFU ml^{-1}. Three heterotrophic bacteria [*Flavobacterium odoratum* Japan Collection of Micro-organisms (JCM, Saitama, Japan) 7458, *Acinetobacter calcoaceticus* JCM 6842 and *Pseudomonas aeruginosa* from JWWA] were mixed and suspended in 250 ml PBS solution to make 60 000 CFU ml^{-1}. Each coliform bacterium and the heterotrophic suspension were mixed in equal volumes. The mixed bacterial suspension was inoculated into 10 tubes of two media and incubated at 35°C. The positive reaction was observed at 20, 24, 28 and 48 h. The experiments were done in duplicate for each coliform bacterium.

Evaluation procedure for untreated and chlorinated natural water

The natural water samples were collected by eight waterwork stations following the instructions issued by JWWA between September (average temperature: 24·4°C at Tokyo) and October (average temperature: 20·1°C at Tokyo) 1998. Ten-litre water samples were collected in sterilized bottles and were kept in the dark and at a cool temperature (5°C) until examination. The examinations were carried out within 48 h of collection. Each water sample was tested in duplicate on the same day. The water samples were put into sterilized 3000 ml Erlenmeyer flasks and stirred with a magnetic stirrer at 20 ± 1°C. The following procedure was carried out in accordance with the instructions of JWWA. NaOCl solution was added to give a concentration of 0·2 mg l^{-1} for lake-waters and 0·5 mg l^{-1} for river-waters. The sampling time after the addition of NaOCl was 20, 60, 180, 300 and 1800 s. Each water sample was then put into sterilized 300 ml Erlenmeyer flasks containing 1 mol l^{-1} of sodium thiosulfate. Total coliforms and *E. coli* in each of the 16 untreated and 80 chlorinated natural water samples (total 96 water samples) were then estimated using EC-Blue-10 and Colilert-MPN (Colilert, IDEXX Laboratories, KK, Tokyo,

Japan) by the five-tube, five-dilution MPN method. Each medium was incubated at 36 ± 1°C. The results were read at 24 h for total coliforms and *E. coli*. The presence of coliforms using EC-Blue-10 was identified by the development of a blue-green colour in an initially light yellow coloured solution and the presence for *E. coli* was identified by the development of fluorescence at 366 nm in the same vessel. For Colilert-MPN, the development of a yellow colour indicated the presence of coliforms and fluorescence on exposure to long-wavelength UV light denoted the presence of *E. coli*.

Isolation and identification

The presence of total coliforms was confirmed by identifying the bacterial isolate(s) to species level from at least one positive EC-Blue-10 and Colilert-MPN tube per row according to the method of Edberg *et al.* (1988). The isolation of bacteria was carried out by streaking onto Levine–Eosin Methylene Blue agar (L-EMB, Becton, Dickinson and Company). Colonies with a typical green metallic sheen, representative of each morphology present, were picked and re-streaked on XM-G agar (the agar medium containing 5-bromo-6-chloro-3-indoxyl-β-D-galactopyranoside (Magenta-Gal) and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, cyclohexylammonium salt (X-Gluc), Nissui Pharma.) and incubated for 24 h at 35°C. Presumptive identification for coliforms and *E. coli* was confirmed by Magenta-Gal and X-Gluc reactions on XM-G agar, respectively. Bacterial isolates were inoculated onto plate count agar to confirm the purity of cultures. The isolates for identification were selected after due consideration of geographical differentiation, sampling time and colony morphology on L-EMB and XM-G agar. Gram-negative rods were identified by API 20E system (bioMerieux Japan Ltd, Tokyo, Japan) and ID TEST EB-20 (Nissui Pharma.) (Kodaka *et al.* 2004). The identification of all isolates was also confirmed by standard methods.

Tests for microbial and physical properties

The tests were done according to the Japanese Standard Methods for Examination of Water (JWWA 1993). The standard plate count (SPC) using plate count agar incubated at 36 ± 1°C for 24 ± 2 h and the heterotrophic plate count (HPC) using PYG agar (g l^{-1}: peptone 2·0, glucose 0·5, yeast extract 1·0, agar 15, pH 7·0 ± 0·1) incubated at 20 ± 1°C for 7 days were carried out before adding NaOCl. The residual chlorine was measured using the *N,N*-diethyl-*p*-phenylenediamine method. The turbidity was measured with a turbidimeter (ANA-7S Tokyo, Koden, Tokyo, Japan) comparing the sample with a kaolin turbidity standard solution [1 mg of kaolin in 1000 ml of distilled water has a turbidity of approx. one nephelometric turbidity units (NTU)]. The water temperature was measured with Celsius liquid-in-glass thermometer. The pH was measured with a pH meter (HM-60V, TOA Electronics, Tokyo, Japan) with a glass electrode.

Statistical analysis

Total results for the 96 sets of MPN data were calculated as $\log_{10}$ MPN of total coliforms and *E. coli* 100 ml^{-1} of water samples. Statistical calculations were carried out with the MICROSOFT EXCEL 2000 statistics package. The statistical analysis consisted of regression analysis and paired *t*-test for the MPN data. The chi-square test and Cohen's kappa for homogeneity of presence/absence results were compared using 2400 tubes of EC-Blue-10 and Colilert-MPN, respectively. All statistical analyses were performed with a level of significance of 0·05. The data were also analysed according to the ISO 17994 (2004) for the establishment of equivalence between EC-Blue-10 and Colilert-MPN methods, prescribes calculation of 100-times the logarithmic (ln) difference. The evaluation of equivalence is based on the mean and the expanded uncertainty derived from the standard uncertainty of the mean.

Results

Effect of media for coliform bacteria from chlorinated water sample

No difference between 24 and 48 h for positive reactions were observed among four media. Therefore, we have compared the cultures for positive reaction at 24 h incubation. A total of 30 tubes were examined at each chlorine treatment time. For the detection of *E. coli* ATCC 11775 after chlorine treatment for 15, 30, 60, 120 and 300 s, positive X-Gal tubes of EC-Blue-10 were 6, 4, 9, 3 and 0, respectively, and positive ONPG tubes of Colilert-MPN were 11, 4, 11, 1 and 1, respectively. Positive MUG tubes of EC-Blue-10 were 6, 4, 9, 3 and 0, respectively, and positive MUG tubes of Colilert-MPN were 10, 2, 11, 1 and 1, respectively. The positive gas production tubes of LB were 19, 15, 18, 4 and 3, respectively. The positive gas production tubes of BGLB were 16, 10, 16, 3 and 0, respectively. For the detection *Cit. freundii* ATCC 8090, positive X-Gal tubes of EC-Blue-10 were 29, 29, 21, 7 and 1, respectively, and positive ONPG tubes of Colilert-MPN were 30, 30, 25, 7 and 5, respectively. The positive gas production tubes of LB were 30, 30, 13, 12 and 4, respectively. The positive gas production tubes of BGLB were 27, 12, 3, 3 and 0, respectively. For the detection of *Ent. cloacae* ATCC 13047, positive X-Gal tubes of EC-Blue-10 were 15, 7, 4, 2 and 0, respectively, and positive ONPG

tubes of Colilert-MPN were 6, 3, 4, 1 and 0, respectively. No positive tubes for gas production in LB and BGLB were observed. For the detection of *Kl. pneumoniae* ATCC 13883, positive X-Gal tubes of EC-Blue-10 were 27, 27, 26, 9 and 12, respectively, and positive ONPG tubes of Colilert-MPN were 3, 5, 3, 1 and 1, respectively. The positive gas production tubes of LB were 25, 29, 28, 20 and 23, respectively. The positive gas production tubes of BGLB were 19, 21, 23, 14 and 12, respectively. MUG reaction in each medium was not observed with *Cit. freundii* ATCC 8090, *Ent. cloacae* ATCC 13047 and *Kl. pneumoniae* ATCC 13883.

Heterotrophic bacterial influence to detect coliforms and *E. coli*

The heterotrophic bacteria at 10^4 CFU level found in these samples appeared neither to interfere with coliforms and *E. coli* detection nor to account for the differences between EC-Blue-10 and Colilert-MPN in coliforms and *E. coli* detection. Mixtures of the heterotrophic bacteria did not result in false-negative analyses.

Untreated and chlorinated natural water samples

A total of 96 MPN tests for each water sample and each NaOCl exposure time were carried out using EC-Blue-10 and Colilert-MPN. The results of the regression analyses for total coliforms are shown in Table 1. Median $\log_{10}$ MPN 100 ml^{-1} ± standard deviation (SD) for total coliforms with EC-Blue-10 and Colilert-MPN were 2·11 ± 1·32 and 2·23 ± 1·38, respectively. The regression coefficient, slope and intercept between EC-Blue-10 and Colilert-MPN were 0·91, 0·96 and –0·0012, respectively (Table 1). The results of the regression analyses for *E. coli* are shown in Table 1. Median $\log_{10}$ MPN 100 ml^{-1} ± SD for *E. coli* with EC-Blue-10 and Colilert-MPN were 0 ± 0·91 and 0 ± 0·89, respectively. The regression coefficient, slope and intercept between EC-Blue-10 and Colilert-MPN were 0·89, 0·85 and 0·084, respectively. The means of MPN results for total coliforms and *E. coli* with EC-Blue-10 and Colilert-MPN were not statistically significantly different ($P > 0·05$) by paired *t*-test. For all comparisons, the slope and intercept values, as determined by linear regression analysis, were close to 1·00 and 0·00, respectively. The presence/absence results using 2400 tubes of EC-Blue-10 and Colilert-MPN were compared. For the coliform test, the results were 959 positive tubes of EC-Blue-10 and 925 positive tubes of Colilert-MPN (Table 2). For the *E. coli* test 208 tubes were positive using EC-Blue-10 and 217 tubes were positive using Colilert-MPN (Table 2). These results indicated no significant difference between the two media using the chi-square test ($P > 0·05$). The substantial agreements between the two kits obtained using Cohen's kappa were 0·79 for total coliforms and 0·72 for *E. coli*. Table 3 shows statistical evaluations of the equivalence of the two methods for total coliforms and *E. coli* according to ISO 17994 (2004). Samples were excluded from calculations when both methods gave zero (0, 0). The expanded uncertainty was derived from the standard uncertainty of the mean by using the coverage factor $k = 2$. The evaluation for results of the comparison and the confidence interval of the expanded uncertainty around the mean was calculated by computing the lower limit (x_L) and upper limit (x_H). The x_L and x_H for total coliforms and *E. coli* were –3·0 and 51·6 and –44·9 and 44·3, respectively. Assuming that the maximum acceptable deviation (D) has been chosen as D = 10%. The means of relative difference for total

Table 1 Parameters of each test for total coliforms and *Escherichia coli* from water samples*

	Total coliforms		*E. coli*	
Parameters	EC-Blue-10	Colilert-MPN	EC-Blue-10	Colilert-MPN
No. of tested samples	96	96	96	96
No. of positive samples	93	87	37	41
Median ($\log_{10}$ MPN/100 ml)	2·11	2·23	0	0
SD ($\log_{10}$ MPN/100 ml)	1·32	1·38	0·91	0·89
95% Confidence limit	0·26	0·28	0·18	0·18
Regression coefficient	0·91		0·89	
Slope	0·96		0·85	
Intercept	–0·0012		0·084	
t†	1·75		–0·17	
df	95		95	

*Include 16 untreated and 80 chlorinated water samples.
†Paired *t*-test at the significance level ($P = 0·05$).

Table 2 Comparison of presence/absence results from 2400 tubes for coliforms and *Echerichia coli*

	Coliforms				*E. coli*			
Test kit	Pr*	Ab†	*k*‡	*P*§	Pr*	Ab†	*k*‡	*P*§
EC-Blue-10	959	1441	0·79	0·62	208	2192	0·72	0·10
Colilert-MPN	925	1475			217	2183		

*Pr, presence.
†Ab, absence.
‡*k*, Cohen's kappa value.
§*P*, *P* value by chi-square.

Table 3 Statistical evaluation of the equivalence of the two MPN methods for total coliforms and *Escherichia coli* according to ISO 17994 (2004)

	No. of Samples	n_0*	n†	Mean relative difference	SD	Expanded uncertainty range x_L	Expanded uncertainty range x_H	One-sided evaluation
Total coliforms	96	1	95	24·3	133·0	−3·0	51·6	Inconclusive
E. coli	96	47	49	−0·3	156·0	−44·9	44·3	Inconclusive

*n_0, number of samples excluded because of zero.
†n, number of samples retained for analysis.

Table 4 Statistical evaluation of P/A results according to ISO 17994 (2004)

	*n*A*	*n*B†	x^2‡
Coliform	147	101	8·53
E.coli	49	63	1·75

**n*A, the number of samples where EC-Blue-10 was positive and Colilert-MPN negative.
†*n*B, the number of samples where EC-Blue-10 was negative and Colilert-MPN positive.
‡x^2, Poisson index of dispersion.

coliforms and *E. coli* were 24·3 and −0·3, respectively. The evaluations for total coliforms and *E. coli* in accordance with one-sided evaluation of ISO 17994 (2004) were both 'inconclusive' because the data were insufficient for decisions. Table 4 shows statistical evaluation of P/A results for coliform and *E. coli* according to ISO 17994 (2004). The values of the Poisson-index of dispersion (x^2) for coliform and *E. coli* were 8·53 and 1·75, respectively. EC-Blue-10 and Colilert-MPN methods were considered to be 'different' for coliform, however both methods were considered to be 'not different' in accordance with evaluation for two P/A methods of ISO 17994 (2004).

Bacterial isolates

Table 5 shows species of Gram-negative isolated from untreated and chlorinated water samples. The total number of isolates for identification from EC-Blue-10 and Colilert-MPN were 41 and 46, respectively. The coliforms (excluding *E. coli*) that were isolated from water samples were *Cit. amalonaticus*, *Ent. agglomerans*, *Ent. cloacae*, *Ent. intermedium*, *Kl. Pneumoniae* and *Serratia marcescens*. There were mixed cultures of total coliforms present in both EC-Blue-10 and Colilert-MPN tubes.

Relation between coliforms and bacterial counts

Table 6 shows the microbiological and physical properties of each sample during the experiments. The HPC ranged from 3550 to 140 500 CFU ml^{-1} with PYG agar and the SPC ranged from 1305 to 141 000 CFU ml^{-1} with plate count agar. No relationship was noticed between SPC, HPC and coliforms.

Table 5 Species of Gram-negative identified

Species	% of all isolates identified by EC-Blue-10	% of all isolates identified by Colilert-MPN
Coliforms		
Citrobacter amalonaticus	5	2
Enterobacter agglomerans	13	1
Ent. cloacae	1	9
Ent. intermedium	3	1
Escherichia coli	40	42
Klebsiella pneumoniae	3	9
Serratia liquefaciens	1	2
Ser. marcescens	10	1
Noncoliforms		
Aeromonas caviae	1	2
Morganella morganii	5	2
Providencia alcalifaciens	1	2
Pseudomonas aeruginosa	8	1
Ps. fluorescens	3	4
Ps. putida	5	15
Proteus vulgaris	1	7

Total isolates for identification from EC-Blue-10 and Colilert-MPN were 41 and 46, respectively.

Discussion

Statistical evaluations of the equivalence of the EC-Blue-10 and Colilert-MPN methods for total coliforms and *E. coli* according to one-sided evaluation of ISO 17994 (2004) were 'inconclusive' for total coliforms and *E. coli*. About 25 additional samples for total coliforms and about 1000 additional samples for *E. coli* would have been sufficient numbers to reach firm decisions. The ONPG test with the ONPG peptone-water medium is preferable for growth of the organisms (Lowe 1962). EC-Blue-10 contains biological material such as peptone for the enhanced growth for bacteria, whereas Colilert-MPN is minimal medium for bacteria. Two ingredients of

Table 6 Microbial and physical properties of each water sample during the experiments

Sampling areas	No. of coliforms isolated		No. of *E.coli* isolated		SPC* (CFU ml^{-1})	HPC† (CFU ml^{-1})	Free Cl^-($mg\ l^{-1}$)	Total Cl^-($mg\ l^{-1}$)	Temp. (°C)	NTU‡	pH
	EC- Blue-10	Colilert- MPN	EC-Blue-10	Colilert-MPN							
A-lake	25	17	4	4	1305	2563	0·1	0·2	21·1	0·6	7·3
B-river	31	38	12	16	21700	120500	0·2	0·4	20·5	2·9	7·4
C-river	26	20	6	6	141000	140500	0·3	0·5	20·1	0·5	7·4
D-lake	46	35	14	5	1118	3550	0·1	0·2	20·8	2·0	8·6
E-river	32	34	8	11	2503	26575	0·2	0·5	20·0	0·8	7·3
F-river	30	26	11	10	16275	71000	0·4	0·5	19·6	2·6	7·3
G-river	33	34	4	7	1973	6700	0·1	0·3	20·1	1·5	7·6
H-river	43	47	13	25	19950	71000	0·1	0·5	20·3	1·1	7·5
Mean	33	31	9	11	25728	55299	0·2	0·4	20·5	1·5	7·6
SD	8	10	4	7	47408	54313	0·1	0·1	0·5	0·9	0·4

Sixteen untreated and 80 chlorinated water samples were tested.
*SPC, Standard plate count.
†HPC, Heterotrophic plate count.
‡NTU: Nephelometric turbidity units.

EC-Blue-10 are also different from Colilert-MPN. Firstly, EC-Blue-10 contains sodium pyruvate as nonenzyme peroxide-degrading compound to increase the detection of chlorine-stressed coliform bacteria (Sartory 1995). Secondly, the KNO_3 in EC-Blue-10 is important for bacteria, as it allows energy production during nitrate respiration (Hadjipetrou and Stouthamer 1965). Bacteria, commonly considered part of the total coliform group were isolated from both EC-Blue-10 and Colilert-MPN tubes. *E. coli* was isolated from tubes with both positive-colour and -fluorescence. *E. coli* was primarily isolated from water samples, followed by *Ent. agglomerans*, *Kl. pneumoniae* and *Serratia marcescens*. Every isolate was inoculated into both media to confirm the reactions. No different reactions were observed between EC-Blue-10 and Colilert-MPN. There did not appear to be a significant difference in the distribution of bacterial species in either medium. The HPC on most samples were higher than the SPC. Only one sample, C-river, had almost the same microbiological count (Table 6). The results of heterotroph interference study and the results in Table 6 support the notion that heterotrophic bacteria do not interfere with the detection or enumeration of total coliforms and *E. coli* by the EC-Blue-10. After the addition of NaOCl, total coliforms were detected from the H-river water sample using both methods. The microbial and physical properties of this water sample were not significantly different from the other water samples (Table 6). We have not investigated why total coliforms were detected after the addition of NaOCl. There was a concern that bacteria other than *E. coli* might exhibit fluorescence. No false-negative results were observed in this study. However, we did find false-positive results, with β-glucuronidase positive *Staph. warneri* being isolated from EC-Blue-10 and pyoverdin positive *Ps. putida* being isolated from Colilert-MPN. *Staph. warneri* could be resistant to 0·1 g SDS l^{-1} in EC-Blue-10 (Kodaka *et al.* 1995). We agree with Edberg *et al.* (1988) that each test was limited by design to drinking water distribution samples and the user should first establish the efficacy of the test in each water sample. A weak fluorescent reaction for MUG test can be read in the aqueous phase of the medium. A disadvantage of EC-Blue-10 was that it was difficult to read a weak-positive blue colour, because the base colour of EC-Blue-10 is light yellow. However, the medium in EC-Blue-10 was developed primarily for the rapid growth of *Enterobacteriaceae* (Kodaka *et al.* 1995). If coliforms were present in the water sample, they could grow sufficiently. Therefore, it would be very rare to observe a weak reaction and if a weak reaction was observed, it could be confirmed by comparison to the EC-Blue-10 comparator.

In conclusion, EC-Blue-10 gave results that were almost statistically equivalent to the DST method currently accepted by the Ministry of Health, Labour and Welfare of Japan. Therefore, the EC-Blue-10 is as useful as the DST method for the detection of coliforms and *E. coli* in temperate humid climate zone water. However, the water samples tested were very limited in this evaluation of EC-Blue-10 and therefore, it is recommended that a more extensive evaluation of EC-Blue-10 be undertaken.

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Microscopy AND Microanalysis

An Instant Cell Recognition System Using a Microfabricated Coordinate Standard Chip Useful for Combinable Cell Observation with Multiple Microscopic Apparatuses

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Abstract: Disposable coordinate standard (CS) chips were fabricated by the ejection of melted polystyrene into a metal mold. The CS chip surface was divided into four parts different in height and width. The edge lines of these parts could be recognized as straight lines 2 μm in width in the microscope view and used as the X and Y axes for the culture dish. The CS chip was attached on the bottom of a culture dish outside. Then the dish was set on the microscope stage and moved by means of a motorized automatic stage. The X-Y coordinates of many single-cells in a culture dish were registered, respectively. Once registered, any single-cell could instantly be brought to the center of the microscope view even after displacing the dish from the stage for a while and setting it again on the stage. Therefore, experimenters can easily search any single-cell in any culture dish on any microscope at any time. Such a system is remarkably useful for various modes of single-cell experiment and named "Suguwaculture," which means "instantly" ("sugu" in Japanese) + "recognizable" ("wakaru" in Japanese) + "culture" (during culture).

Key words: instant cell recognition, coordinate standard chip, X-Y coordinates registration, combinable observation on multiple microscopes, Suguwaculture, single-cell experiment, high throughput microinjection, microscopic image analysis

Introduction

Recently, an enormous amount of information has been accumulated about genomes of various species, and many efforts have been made to cluster it and to give annotations to respective genes (Ashburner et al., 2000; Overbeek et al., 2005). To certify the annotated functions of respective genes, it is necessary to apply the genes of interest to target viable cells and to analyze their responses. Therefore, much interest has now been paid to single-cell experiments. The term "single-cell" means an isolated single cell (one egg cell of vertebrates or insects, one blood cell, one somatic cell separated from tissue by enzymatic treatment, etc.) and a particular target single cell in multicell system (one epidermis cell in skin, one liver cell in a small chip of liver, one leaf cell in a small chip of leaf, etc.) as well. In single-cell experiments, the microscopic observation of each single-cell and its manipulation should be repeated many times every day. Experimenters must continue time-consuming and labor-intensive work, because it is difficult to find each target cell of interest among many other cells within a short time.

Formerly, we developed a single-cell manipulation supporting robot (SMSR) (Matsuoka et al., 2005; Matsuoka & Saito, 2006) to realize higher throughput microinjection. Microinjection includes not only the insertion of a micropipette into a target cell but also various associated work such as X-Y stage manipulation, microscope focus adjusting, the selection and transportation of a cell, the exchange of a micropipette, and the record of the microscopic images of the cell. This work may be classified into two categories: actions performed by microscopic observation (on-microscope actions) and actions performed by taking the eye off the microscope (off-microscope actions) (Matsuoka et al., 2006). Frequent

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changes of these on-microscope and off-microscope actions are annoying and time-consuming. The concept of SMSR is to let an operator concentrate his/her attention only on the microinjection by facilitating other associated actions.

To realize this concept, an X-Y coordinates registration system for each cell was essential. Formerly, various plates with multiple microwells or multiple pores were devised (Meek & Pantano, 2001; Yang et al., 2002; Maruyama et al., 2005; Park et al., 2005; Retting & Folch, 2005; Yasukawa et al., 2005). There are commercially available microscope slides and culture dishes with special scale bars or grid patterns for X-Y coordinates registration (Grid seal, Asahi Techno Glass-IWAKI, Chiba, Japan; Cellocate, Eppendorf, Hamburg, Germany) and stage control systems for time-lapse measurements (AQUA-COSMOS, Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan; MetaMorph, Molecular Device, Downingtown, PA). The slide for a laser-scanning cytometer (LSC) should be the most elegant one (Kamentsky & Kamentsky, 1991) and can be applied to multiple LSCs such as LSC101 (Olympus, Tokyo, Japan), iCyte and iCys (CompuCyte, Cambridge, MA). Those devices seemed to be useful for the X-Y coordinates registration and were applied to some cases of single-cell experiments. However, they were designed for the specific apparatus and not supposed to be used in other apparatuses. That was one reason that a novel device common to various apparatuses was necessary. Another and more critical reason was that those slides and dishes should contact directly with test samples (cells and tissues). Many cell scientists are nervous about their material and surface treatment, because they could influence the growth and physiological properties of test samples. Therefore, it was an intense need to develop a novel X-Y coordinates registration system in which any type of dishes could be used.

To meet this requirement, we previously developed a coordinate glass chip by a photo-resist process, but its cost was too high to be used as disposable chips. Then we intended to develop a more convenient chip at low cost. This study reports a successful result of the production of a plastic chip made by the melted polymer ejection method.

Materials and Methods

Fabrication of the Coordinate Standard Chip

The coordinate standard (CS) chip was produced by the pressure ejection of melted polymer into a metal mold. The Young's modulus, the fracture toughness, and the cost of various polymers were compared, and finally polystyrene was selected as the best one. The metal mold was machined according to the suggestions and advice of several experts. An adhesive tape made of transparent film was pasted on the chip. The CS chip was packaged with plastic double sheet at one chip per partition.

The Suguwaculture System

A microscope used in this study was an inverted microscope IX-71 (Olympus, Tokyo, Japan). Microscopic images were captured with an ORCA-ER CCD camera (Hamamatsu Photonics) and analyzed by AQUA-C Imaging (Hamamatsu Photonics). A cross reticule was set in an eyepiece of the IX-71. An automatic stage system was a part of the SMSR (Matsuoka et al., 2005). A single-cell manager previously developed for SMSR was updated to fit the present system. The system was also combined with another microscope system, for example, the inverted laser scanning microscope (LSM510 system [Axiovert and LSM510], Carl Zeiss, Jena, Germany) for high quality image analysis.

Microinjection Experiments

Fluorescein dextran (MW 70,000) (D-1823, Invitrogen-Molecular Probes, OR) was used to demonstrate consecutive microinjection into multiple mouse embryonic stem (ES) cells. The fluorescent dye (5 $\mu g/\mu l^{-1}$) was microinjected by pressure.

Culture of Mouse ES Cells

Feeder free mouse ES cells were provided by H. Niwa (Center for Developmental Biology, Riken Institute, Kobe) and cultured at 37°C in the absence of feeder cells in Glasgow minimal essential medium (GMEM) (G5141; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (04-001-1A; Biological Industries, Belt-Haemek, Israel), 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 1× nonessential amino acids (NEAA) (11140, Invitrogen-Gibco, Grand Island, NY), and 1000 $U ml^{-1}$ of leukemia inhibitory factor (LIF) (ESG1107; Chemicon International, Temecula, CA) on gelatin (TYPE B from Bovine Skin, G9391; Sigma-Aldrich)-coated polystylen culture dishes (35 mmϕ) (3000-035; Asahi Techno Glass-IWAKI, Chiba, Japan) (Smith, 1991). A cell suspension (2 ml) containing 5000–10,000 ES cells was placed on the bottom of the culture dish and covered with mineral oil and then incubated at 37°C.

Culture of Tobacco Cells

Cultured tobacco cell line BY-2 derived from *Nicotiana tabacum* L. cv. Bright Yellow-2 (Kato et al., 1972) was cultured in Linsmaier-Skoog medium (30 $g l^{-1}$ sucrose, 200 $mg l^{-1}$ potassium phosphate monobasic, 100 $mg l^{-1}$ myo-inositol, 1 $mg l^{-1}$ thiamine hydrochloride, 4.41 $g l^{-1}$ Murashige and Skoog Plant salt Mix [2633022; MP Biomedicals, Illkirch, France]) supplemented with 0.2 $mg l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) (LSD medium) at 28°C in the dark with rotation at 130 rpm. The cultured cells were transferred into a fresh medium every 7 days. After a 4-day culture since the last transfer, the BY-2 cells were used in subsequent experiments.

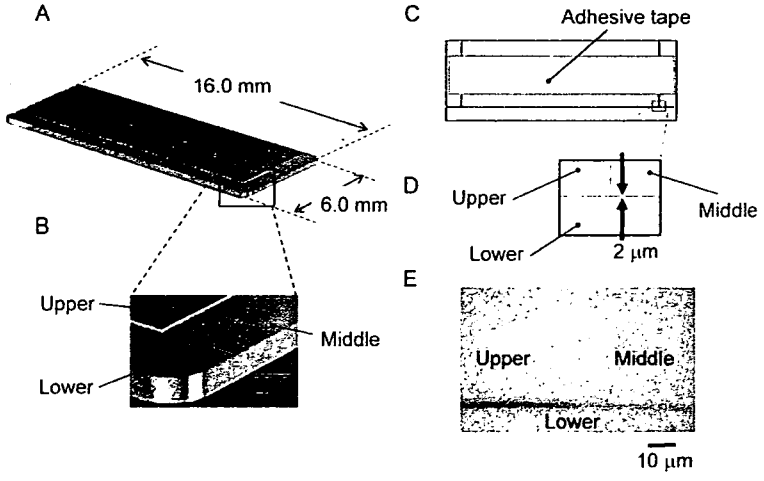

Figure 1. Surface structure and size of a CS chip. Three sizes are provided: 16 mm × 6 mm × 0.3 mm (**A**), 16 mm × 10 mm × 0.3 mm, and 40 mm × 23 mm × 0.3 mm. Adhesive tape is pasted on the top surface of the chip (**B,C**). Microscopic view of the edge lines formed on the top surface (**D,E**).

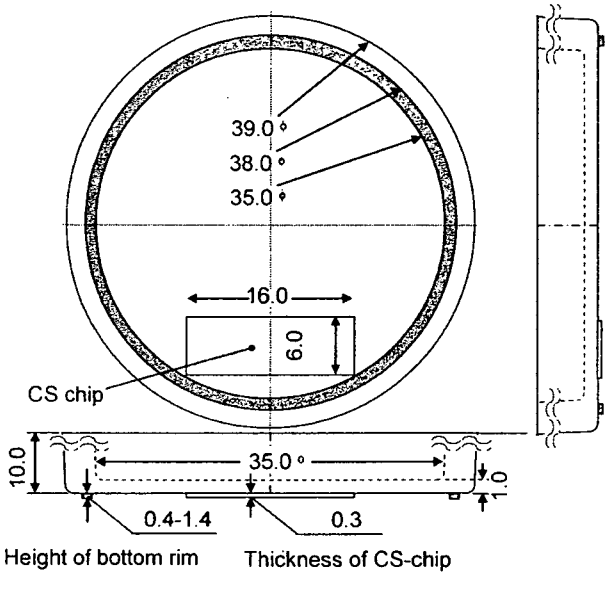

Figure 2. CS chip attached on a 35 mmϕ culture dish (3000-035, Asahi Techno Glass-IWAKI) (Figure unit: mm).

Results and Discussion

A plastic CS chip was fabricated as depicted in Figure 1. The chip size was 16.0 mm × 6.0 mm. The chip surface was divided into four parts different in height and width (Fig. 1A). The edge lines of these parts could be recognized as straight lines 2 μm in width in the microscope view (Fig. 1D,E). The chip was attached on the bottom of a culture dish outside (Fig. 2). The culture dish depicted in Figure 2 is a popular one used in our laboratory. The thickness of the CS chip was made no thicker than 0.3 mm because the height of bottom rim of commercially available culture dishes ranges from 0.4 to 1.4 mm.

Then the dish was set on the microscope stage (Fig. 3). The stage can be driven by handling a joystick controller. A foot switch was used to register the X-Y coordinates. The software developed previously for SMSR was properly upgraded and applied to the present system. Figure 4 depicts how to register the X-Y axes fixed to respective culture dishes. A culture dish is set in the dish holder on the automatic stage (Fig. 4). The coordinates fixed to the automatic stage are U-V axes. The experimenter drives the joystick controller so that the point P of the CS chip comes to the microscopic view center (MVC). The point P is registered as the origin by clicking the foot switch. Then the experimenter drives the joystick controller so that the point Q of the CS chip comes to MVC. The line PQ is registered as the X-axis by clicking the foot switch. The Y-axis is defined automatically as a line that passes the origin (P) at right angle to the X-axis.

The cell coordinates registration follows the X-Y axes registration. The experimenter selects any one cell and drives the joystick controller so that the cell (e.g., $Cell_1$ in Fig. 5) comes to MVC. By clicking the foot switch, the X-Y coordinates of the cell are registered and at the same time the U-V coordinates are calculated automatically according to the formulas depicted in Figure 5. In the same way, the cell search and the registration of its X-Y coordinates are performed successively. The number of the cell is registered consecutively and displayed in the column on "Cell number" (Fig. 6). The relative position of the registered cell is plotted in a circle representing the culture dish. When different kinds of cells are concerned, they can be differently

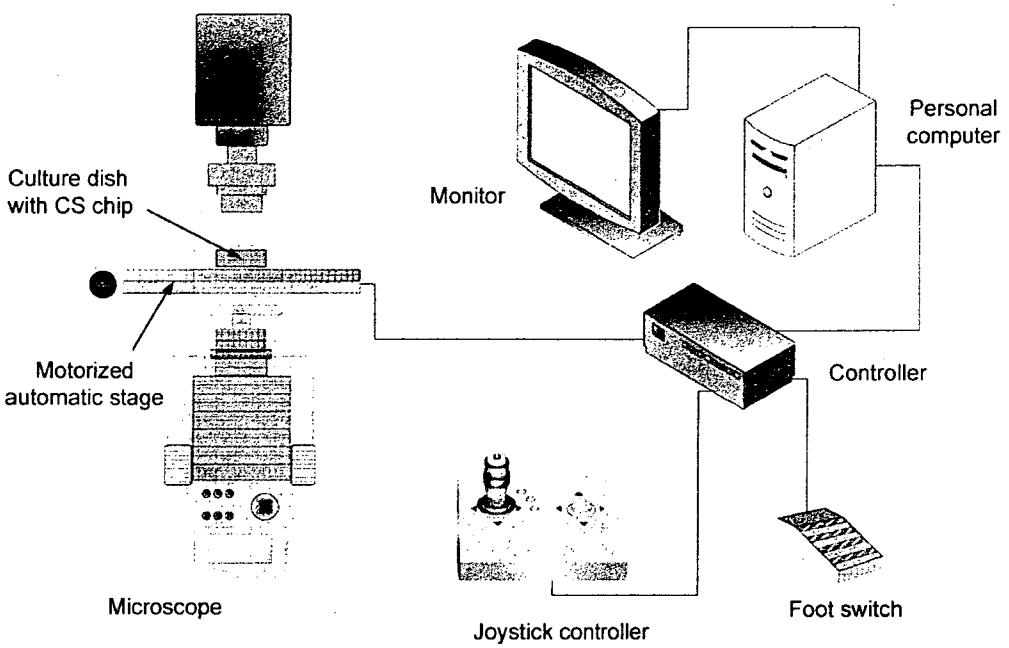

Figure 3. Schematic diagram of a Suguwaculture system.

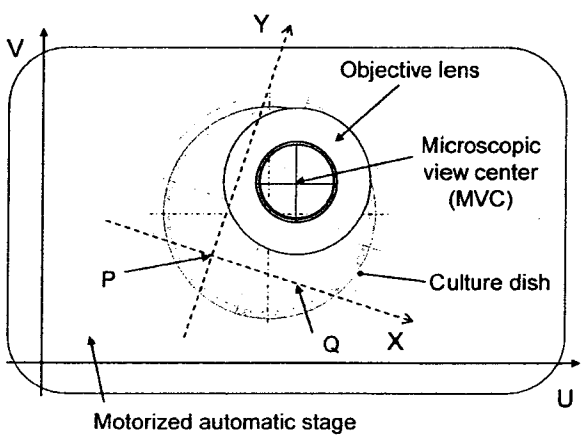

Figure 4. Realtive position of a culture dish, an automatic stage, and an objective lens. A culture dish with a CS chip is set on the microscope stage, and the points P and Q are adjusted at the MVC in this order to register the origin and the X axis. Microscopic view (center area of the objective lens) is illustrated on the nonscale.

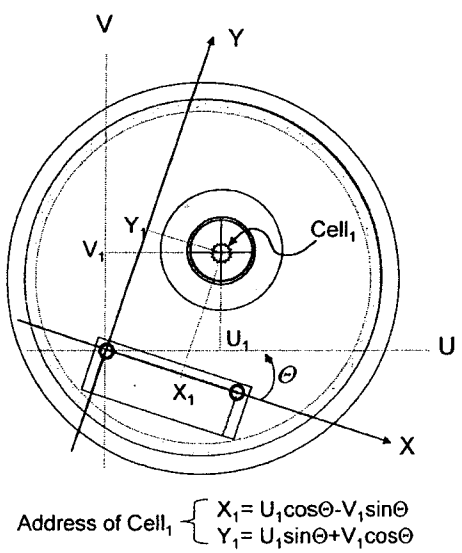

Figure 5. Registration of the X-Y coordinates of a cell. (U_1, V_1) is the distance from the origin automatically measured from the driving distances of two pulse motors. The motors drive the stage in the U and V directions, respectively. X_1 and Y_1 are calculated automatically from U_1 and V_1 according to the formula.

marked in the "Cell kind" column. The "Remarks" column is used to record the conditions and results of successive experiments.

When a single-cell experiment such as microinjection or image capture is to be performed successively, an experimenter can call $Cell_i$, $Cell_{i+2}$ . . . $Cell_{i+n}$ to the MVC consecutively only by clicking the foot switch. Alternatively, the experimenter can select $Cell_i$ directly by placing the mouse pointer at the ith "Cell number" (Fig. 6) and clicking the mouse. Such a rapid cell search function was remarkably useful for various single-cell experiments.

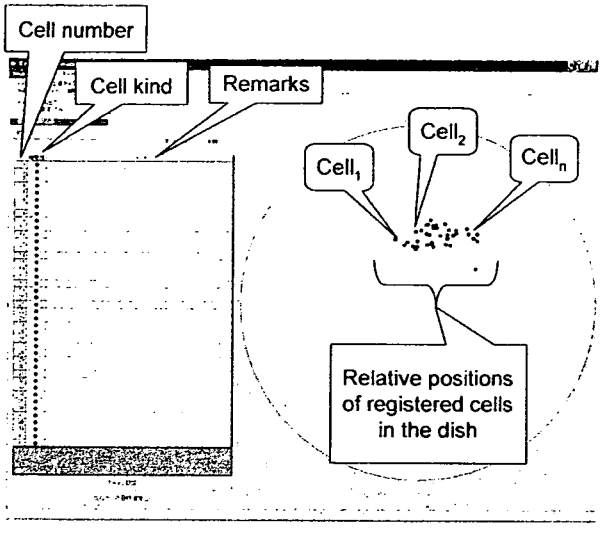

Figure 6. Typical image of a TV monitor displaying the list and the site map of registered cells.

Even when the dish has to be displaced from the automatic stage, and after a while, it is set on the stage again, this rapid cell search function can be used only by the registration of the X-Y axes first before single-cell experiments. Such cases include, for instance, the simultaneous use of multiple culture dishes, the cell culture in a separate incubator, and the image capture with different microscopic apparatuses (Fig. 7).

Figure 8 illustrates the cell images just called to the MVC. The size of a mouse embryonic stem cell is no greater than 20 μm in diameter (Fig. 8A) and its center was registered as the cell position (X_i, Y_i). In the case of tobacco cultured cells line BY-2 (Fig. 8B), the cell size was 50 × 100 μm^2 and the cell center was registered as the cell position (X_j, Y_j). Figure 8C illustrates the consecutive calls of mouse ES cells for the cell registration (a) and microinjection of a fluorescent dye (b) with IX-71, and the cell observation (c) and fluorescent microscopic image capture (d) with the LSM510 system. These cells could be called precisely to the registered cell positions, respectively, and microinjection could be performed rapidly at 200 cells/h (b). Figure 8D demonstrates the consecutive observation of the growth process of single-cells for 6 days. Except for everyday observation time, the dish was placed in an incubator throughout. Three images (a, b, c) were captured from different dishes. Even in case (c), where three single cells lined within the same view, the target cell (indicated by the arrow) could be called to the MVC instantly and easily distinguished from other neighbor cells.

Conclusions

The disposable CS chip has realized high performance of the Suguwaculture system. With this system, the X-Y coordinates data of single-cells can be used in common in time-lapse experiments using multiple apparatuses. The CS chip

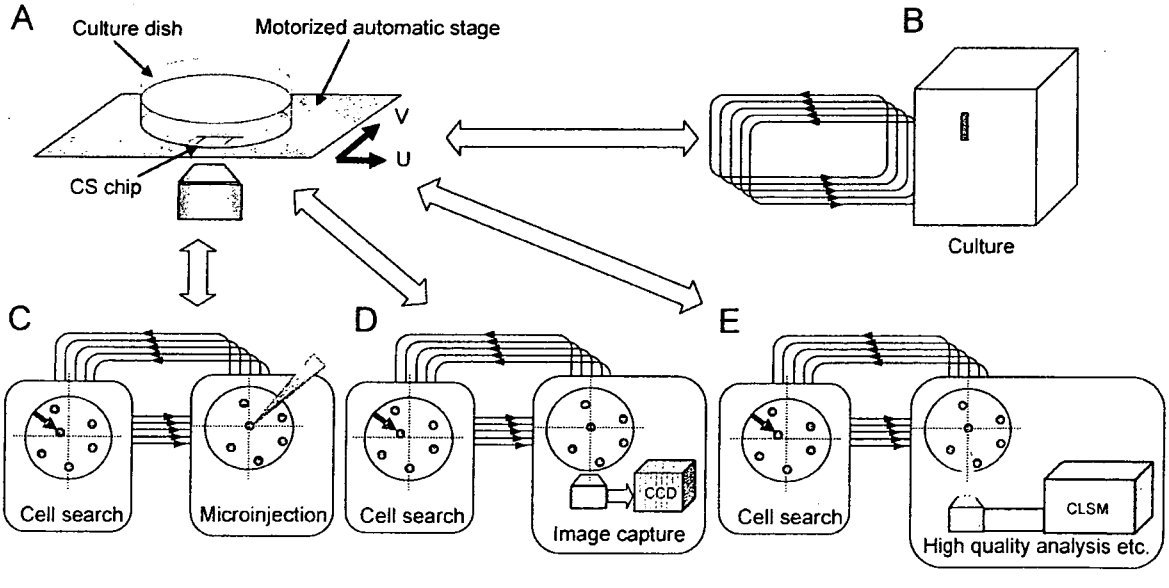

Figure 7. Time-lapse single-cell experiments using multiple apparatuses demonstrated by the Suguwaculture system. **A:** Single-cell experiments such as cell registration and cell manipulation on the automatic stage of an inverted microscope. **B:** Culture in an incubator. **C:** Microinjection. **D,E:** Time-lapse image captures with other optical apparatuses.

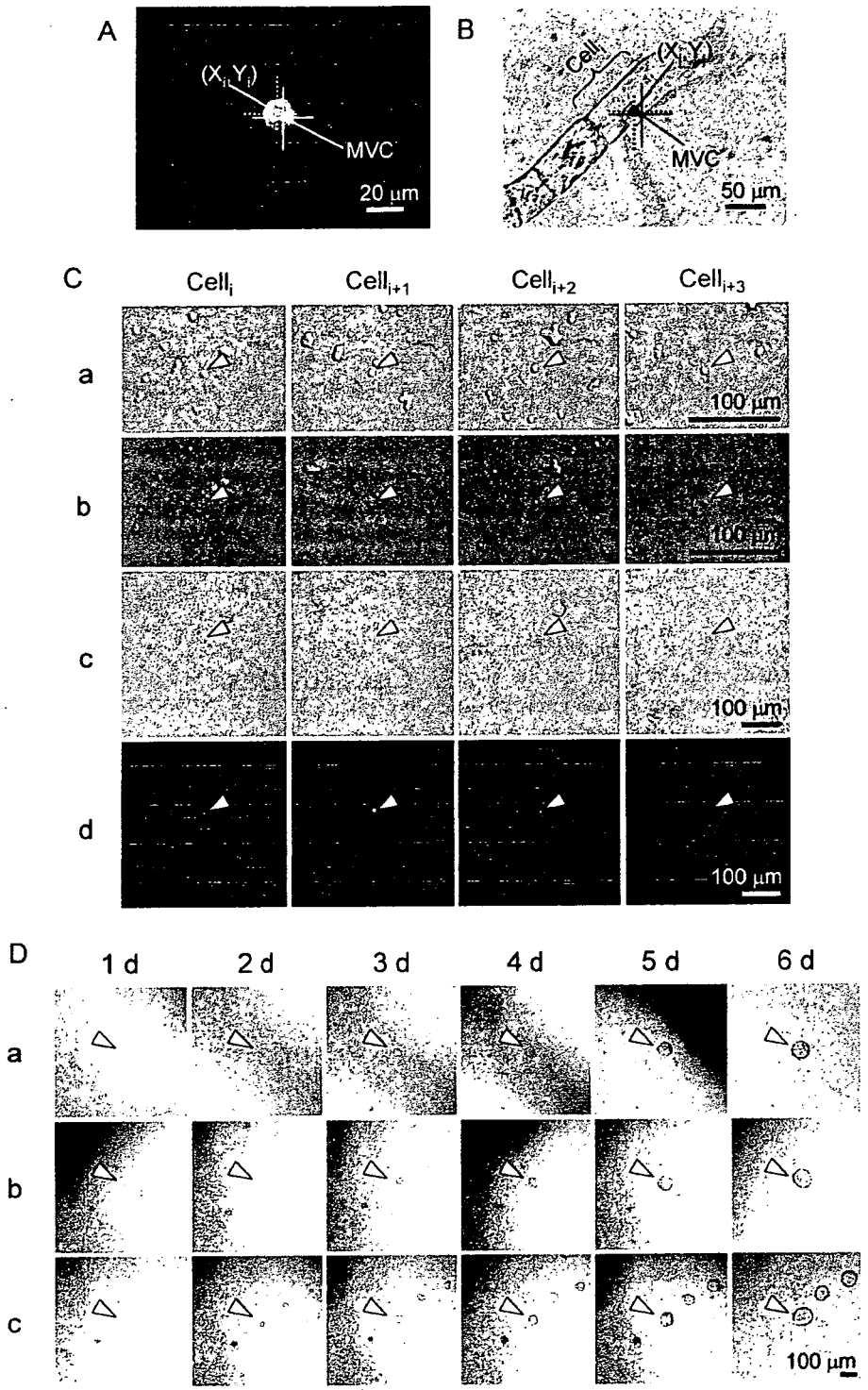

Figure 8. Performance of the instant call of target cells to MVC. **A:** Mouse ES cell. **B:** Cultured tobacco BY-2 cell. **C:** Consecutive registration of multiple mouse ES cells and suuccessive microinjection of a fluorescent dye and their observation with a different optical apparatus: (a) before and (b) after microinjection of fluorescent dye with IX-71, (c) bright field image, and (d) fluorescent image observed with LSM510 system. White arrows indicate target cells. Cell$_i$, Cell$_{i+1}$ . . . Cell$_{i+n}$ indicate a part of consecutive numbers in many cells. **D:** Consecutive observation of the cell growth process. a, b, and c indicate three different single-cells in different dishes. Each single-cell at 1st day grew into a colony 100 μm in diameter at 6th day.

is applicable to any type of dishes produced by many companies. Therefore, the disposable CS chip can be used by many researchers and students who are engaged in various single-cell experiments with different types of culture dishes.

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Humoral Immune Responses Against Norovirus Infections of Children

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In 2 infants with gastroenteritis associated with Norovirus (NoV), serum immunoglobulin (Ig) G, IgM, IgA, and fecal IgA antibody responses against NoV were examined by enzyme-linked immunosorbent assay using 11 different antigenic and genetic types of NoV virus-like particles expressed in insect cells. These two cases were putative primary single NoV infections, because antibodies against NoVs were not detected in acute-phase serums. In one of two cases, long-term excretion of virus RNA for 33 days was observed. Serum IgG responses demonstrated strong seroresponse to the homologous type, and weak seroresponse to the heterologous types within the genogroup. After more than 2 years, the IgG antibody titer remained high to the homologous type and low to the heterologous type within the genogroup. IgM and IgA were specific to the homologous type. IgM was short lived and the serum IgA antibody titer remained low to the homologous type for a long period. These results improve our understanding of the humoral immune response to NoV infection.
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KEY WORDS: norovirus; primary infection; humoral immune response; ELISA; virus-like particle

INTRODUCTION

Noroviruses (NoVs) in the family of *Calicviridae* are the major cause of acute nonbacterial gastroenteritis in all age groups, and NoV infections have occurred worldwide as outbreaks and sporadic cases [Green et al., 2001].

NoVs are a genetically and antigenically diverse group. Genetic analysis of the RNA polymerase and capsid region revealed that human NoVs can be divided into two genogroups, genogroup I (GI) and genogroup II (GII) [Green et al., 1994; Wang et al., 1994]. Recent studies of genotyping on the basis of capsid *N*-terminal/shell (N/S) domain classified NoVs into at least 31 genotypes (14 genotypes in GI and 17 genotypes in GII) [Katayama et al., 2002; Kageyama et al., 2004]. These viruses cannot be grown in cell culture, but the expression of the major capsid protein (VP1) in insect cells resulted in the formation of virus-like particles (VLPs) that are morphologically and antigenically similar to native NoV [Jiang et al., 1992; Lew et al., 1994]. Antigenic analysis using enzyme-linked immunosorbent assay (ELISA) with VLPs and hyperimmune antisera showed that the genetic and antigenic relationship corresponded well [Kobayashi et al., 2000a,b,c; Katayama et al., 2002; Kamata et al., 2005; Hansman et al., 2006], but there were unusual cross-reactivities between certain genogroups and/or genotypes based on the antibody ELISA [Hansman et al., 2006]. Serological studies using these recombinant VLP (rVLPs) have shown a high prevalence and broad responses of NoV-specific antibodies both in children and adults [Parker et al., 1994a, 1995; Noel et al., 1997; Farkas et al., 2003]. IgM, IgA, and IgG serologic responses in adult volunteers and patients also reported [Treanor et al., 1993;

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Gray et al., 1994; Rockx et al., 2005a]. But almost all sera used in previous studies had pre-existing antibodies against many types of NoV. Therefore, it was not clear whether the production of cross-reactivity antibodies emerged after primary infection or by multiple infections during their lifetime. In addition, there is little information about the immune response to NoV infections in infants, including primary infection. To interpret the cross-reactivity of antibodies to NoVs, it is necessary to investigate the immune response to primary NoV infection.

In the present study, serum and fecal samples from infants infected with NoV were examined for specific IgM, IgA, and IgG using ELISA with 11 different genotypes of VLPs. This study describes the cross-reactivity and specific antibody responses in putative primary NoV infection.

MATERIALS AND METHODS

Patients

Fecal or serum samples were collected from 2 infants suffering from nonbacterial gastroenteritis at their medical examination and follow-up in the hospital. After the ethical discussion in this working group, we concluded that this study was ethically acceptable. Informed consent for this study was obtained from their parents. The histo-blood group antigen (HBGA) type of the two infants was unknown. Their development was normal. After recovery from diarrhea, their prognosis was favorable. These two cases were sporadic and not related to each other.

Patient A (male): He had acute gastroenteritis at 2 years in December. The major symptoms were diarrhea and vomiting. The diarrhea was sometimes accompanied by benign afebrile convulsions. He had mild diarrhea for about 6 weeks. During the diarrhea, five fecal and serum samples on the 5th, 12th, 18th, 33rd, and 40th days post-onset were collected. After recovery from diarrhea, one fecal and one serum sample on the 60th day post-onset, and one serum sample at 2 years and 10 months post-onset were collected. Re-infection of NoVs during this long period was unknown.

Patient B (male): He had acute gastroenteritis at 1 year in January. The major symptom was diarrhea. The diarrhea was sometimes accompanied by benign afebrile convulsions. He had mild diarrhea for about 1 week. During the diarrhea, one fecal and one serum sample on the 2nd day post-onset were collected. After recovery from diarrhea, one serum sample on the 105th day post-onset was collected. He did not have gastroenteritis again during the period studied.

Detection of NoVs and Other Viruses in Fecal Specimens

NoVs were detected using RT-PCR with primer pairs, Ando's G1 (SR33, SR48, SR50, and SR52) and G2 (SR33 and SR46) primer sets [Ando et al., 1995] amplifying a 123-bp RNA polymerase region, mon381/mon383 [Noel et al., 1997] amplifying a 322-bp capsid region, and SK primers [Kojima et al., 2002] amplifying a 344-bp capsid N/S region as previously described [Iritani et al., 2000; Seto et al., 2005]. Other gastroenteritis viruses were detected with our laboratory method [Iritani et al., 2003]. Briefly, antigens of group A rotavirus and enteric adenoviruses (serotypes 40 and 41) were tested using commercially available antigen ELISA kits, ROTACLONE, and ADENOCLONE-E, respectively, according to the instructions (Meridian Bioscience, Inc., Cincinnati, OH). Enteroviruses and adenoviruses were tested using cell cultures with Vero and RD-18S cells. The virus-negative samples for group A rotavirus, adenoviruses, enteroviruses, and NoVs were tested using electron microscopy (EM) [Iritani et al., 2000], to directly detect virus particles with a negative stain.

Genetic Analysis of NoVs

Sequencing of RT-PCR products and phylogenetic analysis were performed as previously described [Iritani et al., 2000; Seto et al., 2005]. Genotyping based on the Capsid N/S domain was performed as described by Katayama et al. [2002] and Kageyama et al. [2004].

Expression of VLPs

Eleven VLPs (four genotypes in GI and seven genotypes in GII), expressed in insect cells infected with recombinant baculoviruses carrying the capsid gene, were used for ELISA (Table I). These VLPs were produced in the Department of Virology II, National Institute of Infectious Disease. The expressed capsid antigens were purified by a sucrose gradient followed by CsCl gradient centrifugation and confirmed by EM, as previously described [Kobayashi et al., 2000a,b,c; Tamura et al., 2000; Kamata et al., 2005].

Antibody ELISA for Serum Samples

The wells of 96-well flat-bottom microtiter plates (IMMULON2 HB, Dynex Technologies, Inc., Chantilly, VA) were coated with 100 μl of each VLP (0.5 μg/ml in 0.05 M carbonate–bicarbonate buffer (pH 9.6)), and incubated at 4°C overnight. The wells were then washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T; pH 7.2) using a micro plate washer (S8/12J model, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and were blocked at room temperature for 1 hr with 200 μl of Block Ace (Dainippon Pharmaceutical Co., Ltd.). The wells were washed twice and twofold serial dilutions in PBS-T containing 25% Block Ace (25% BA/PBS-T) of serum samples, starting at a 1:50 dilution, were added to antigen-coated plates. After incubation for 1 hr at 37°C, the wells were washed five times, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (heavy and light chains), IgM (μ-chain), or IgA (α-chain) -specific antibodies (Zymed Laboratories, Inc., South San Francisco, CA) at a dilution of 1:4,000 in 25% BA/PBS-T were added, and the plates were incubated for 1 hr at 37°C. After washing the wells five times, 100 μl of substrate, 0.4 mg/ml of *O*-phenylenediamine (Sigma Chemical Co.

TABLE I. Description of 11 VLPs Used in This Study

Genogroup	Genotype	VLP	Strain	Accession no.	References
GI	GI/1	rSeto	Aichi124/1989/JP (Seto)	AB031013	Kobayashi et al. [2000b]
	GI/2	rFUV	Funabashi 258/1996/JP	AB078335	Tamura et al. [2000]
	GI/3	r645	Kashiwa645/1999/JP	BD011871	Kamata et al. [2005]
	GI/4	rChiba	Chiba407/1987/JP	AB022679	Kobayashi et al. [2000a]
GII	GII/3	r809	Sanbu809/1998/JP	BD011876	Kamata et al. [2005]
	GII/4	rNAV	Narita104/1997/JP	AB078336	Kitamoto et al. [2002]
	GII/5	r745	Ichikawa745/1998/JP	BD011877	Kamata et al. [2005]
	GII/6	rUEV	Ueno7k/1994/JP	AB078337	Tamura et al. [2000]
	GII/7	r10–25	Osaka10-25/1999/JP	BD011881	Kamata et al. [2005]
	GII/12	rChitta	Chitta76/1996/JP	AB032758	Kobayashi et al. [2000c]
	GII/14	rKAV	Kashiwa47/2000/JP	AB078334	Kitamoto et al. [2002]

Ltd., St. Louis, MO) was added and the plates were incubated for 30 min at room temperature. The reaction was stopped with 50 μl of 4N H_2SO_4. Absorbance at 492 nm (A492) was measured with a microplate reader (Multickan MS-UV model, Labsystems OY, Helsinki, Finland). IgG, IgM, or IgA titers were defined as the highest dilution of serum given an A492 that was threefold higher than the A492 of the corresponding antigen control well.

Antibody ELISA for Fecal IgA

Five fecal samples on the 5th, 12th, 18th, 40th, and 60th days from Patient A were used for ELISA to detect VLP-specific IgA. There was not sufficient volume of the other samples for ELISA. Fecal samples were prepared as a 10% (wt/vol) suspension in PBS containing 10% fetal bovine serum (FBS). Each fecal suspension was homogenized, and centrifuged at 12,000*g* for 10 min. The supernatant was used for ELISA to detect VLP-specific IgA.

The plates were coated with VLPs as described above. Twofold serial dilutions of fecal suspensions were made in PBS containing 10% FBS. The diluted suspensions were added to antigen-coated plates; thereafter, the ELISA protocol was performed as described above. Virus-negative fecal samples were included on each plate as a negative control. The sample was considered positive for VLP-specific IgA when the absorbance of the well containing fecal samples from patient A was threefold higher than that of the negative control well.

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study were deposited in DDBJ with the following accession numbers: AB089871, OCS980730 (patient A); AB262773, OCS000564 (patient B).

RESULTS

Detection of the Viruses in Stool Specimens From Two Infants With Gastroenteritis

NoVs were detected in two infants by RT-PCR (Table II). In patient A, who had mild diarrhea for about 6 weeks, four fecal samples on the 5th, 12th, 18th, and 33rd day were NoV-positive, although the sample on the 33rd day produced a thin band (data not shown). Those four RT-PCR products had identical nucleotide sequences. In patients B, who had mild diarrhea, fecal samples on the 2nd were NoV-positive, respectively. These stool specimens were negative to other etiological agents of gastroenteritis. NoV strains from the two infants were classified as GII/4 genotype in the capsid N/S region (Fig. 1). These two NoV strains were closely related, with 100% amino acid identity and 98.8% nucleotide identity in the RNA polymerase region, and 100% amino acid identity and 98.5% nucleotide identities in the capsid region. Pairwise comparison of the capsid N/S region showed that these two NoV strains had 100% amino acid identity and 98.9% nucleotide identities to NAV (GII/4), 73.4–78.7% amino acid identities to the other 6 GII VLP strains, and 60.2% amino acid identities to the 4 GI VLP strains.

Detection of Specific Antibodies to 11 VLPs in the Two Infants

The specific IgG antibody titer to the 11 VLPs is shown in Table II. In two acute-phase serum samples from patient A on the 5th day and patient B on the 2nd day, none of the specific IgG antibodies to all VLPs were detected (<1:50). IgG antibodies to rNAV in patient A showed that the first detection was the 12th day post-onset, and the highest titer was observed on the 33rd to 60th day (1:25,600). IgG antibodies to other five GII VLPs excluding r809 appeared from the 18th to 40th day post-onset and their appearance had a time lag. In a serum sample from patient A at 2 years and 10 months, high IgG titers (1:6,400) to rNAV have persisted, and those IgG titers to the other six GII VLPs were the same or higher than other convalescent-phase serum. In patient B, IgG antibodies to rNAV and other six GII VLPs including r809 were detected with high titer (1:51,200) and low (1:100 – 1:200), respectively. There were no specific IgG antibodies to the four GI VLPs in any serum samples.

Specific IgM antibodies to rNAV were detected in four serum samples on the 12th, 18th, 33rd, and 40th days from patient A (Table III). Two serum samples on the 12th and 18th days had a high titer (1:1,600), and later

TABLE II. Detection of IgG to the 11 Kinds of VLPs in Serum Samples Collected From Infantile Patients by ELISA

Patient (age)	Time post-onset of illness	Symptoms[a]	RT-PCR[b]	Reciprocal of serum dilution										
				Genogroup I				Genogroup II						
				rSeto (GI/1)	rFUV (GI/2)	r645 (GI/3)	rChiba (GI/4)	r809 (GII/3)	rNAV (GII/4)	r745 (GII/5)	rUEV (GII/6)	r10-25 (GII/7)	rChitta (GII/12)	rKAV (GII14)
A (2 years)	5th day	D, V, AC	+	<50	<50	<50	<50	<50	**<50**	<50	<50	<50	<50	<50
	12th day	D	+ +	<50	<50	<50	<50	<50	**800**	<50	<50	<50	<50	<50
	18th day	D, AC	+ +	<50	<50	<50	<50	<50	**12,800**	50	<50	50	<50	<50
	33rd day	D, AC	+w	<50	<50	<50	<50	<50	**25,600**	200	<50	100	50	100
	40th day	D, AC	−	<50	<50	<50	<50	<50	**25,600**	200	50	100	50	100
	60th day	Recovered	−	<50	<50	<50	<50	<50	**25,600**	200	50	100	100	100
	2 years 10 months	Normal	NT	<50	<50	<50	<50	400	**6,400**	400	200	200	200	400
B (1 year)	2nd day	D, AC	+	<50	<50	<50	<50	<50	**<50**	<50	<50	<50	<50	<50
	105th day	Recovered	NT	<50	<50	<50	<50	100	**51,200**	100	100	200	100	100

Bold: homologous type.
[a]D, Diarrhea; V, vomiting; AC, afebrile convulsions.
[b]+, Positive; +w, positive but weak band; −, negative; NT, not tested.

samples had a reduction in the IgM titer. There were no specific IgM antibodies to rNAV in serum samples from patient B. IgM antibodies to the other 10 VLPs were not detected in any serum samples.

Specific IgA antibodies to rNAV were detected in all serum samples excluding two acute-phase sera on the 5th day of patient A and the 2nd day of patient B (Table III). For patient A, three serum samples on the 18th, 33rd, and 40th days had a high IgA antibody titer (1:800–1:1,600) and a serum sample at 2 years and 10 months had a low titer (1:100) to rNAV. IgA antibodies to the other 10 VLPs were not detected in any serum samples excluding a serum sample from patient A at 2 years and 10 months to r809 (Table III).

In the five fecal samples of patient A, specific IgA antibodies to rNAV were detected in three samples collected after the 18th day post-onset (Table III). IgA antibodies to the other 10 VLPs were not detected in any fecal samples.

DISCUSSION

From the detection of NoV-specific antibodies in the two patient sera, it appeared that these two patients were putative primary single infections of NoV because antibodies against NoVs were not detected in acute-phase sera. These two cases showed three distinct features compared to the usual NoV-associated gastroenteritis; obstinate mild diarrhea for 1 and 6 weeks, long-term excretion of virus RNAs from patient A for 33 days, and benign afebrile convulsions. The detection of NoVs in patient A indicated that his diarrhea was related to NoV infection up to 33 days, but the direct relation between diarrhea and NoV infection is then unclear, because NoV was not detected from fecal samples of diarrhea on the 40th day post-onset. In a recent study, Rockx et al. [2002] showed that children under 1 year had a tendency toward long-term duration of diarrhea up to the 28th day and excretion of the virus gene up to the 22nd day. Some reports also showed the long-term duration of diarrhea in NoV infections in patients under 2 years [Sakai et al., 2001; Tsugawa et al., 2006]. Our previous study showed that benign afebrile convulsion-associated diarrhea in NoV infections was observed in 6.7% of children under 2 years [Iritani et al., 2003]. Primary NoV infection or age under 2 years may be related to those three distinct features, persistent diarrhea, viral excretion, and afebrile convulsion. Therefore, these two cases in this study were considered general infection cases with NoVs.

Our data showed that the kinetics of serum antibody responses for NoV infection as follows, the peak of IgM in 5th to 12th day, decrease in 18th to 33rd day, and disappearance in 40th to 60th day; the peak of IgA in 12th to 18th day, decrease in 40th to 60th day, and persistence for a long period with low titer; the peak of IgG in >18th day, and persistence for a long period with high titer. This kinetics of serum antibody responses is similar to those seen in previous studies [Erdman et al., 1989; Gray et al., 1994; Brinker et al., 1998, 1999]. Fecal

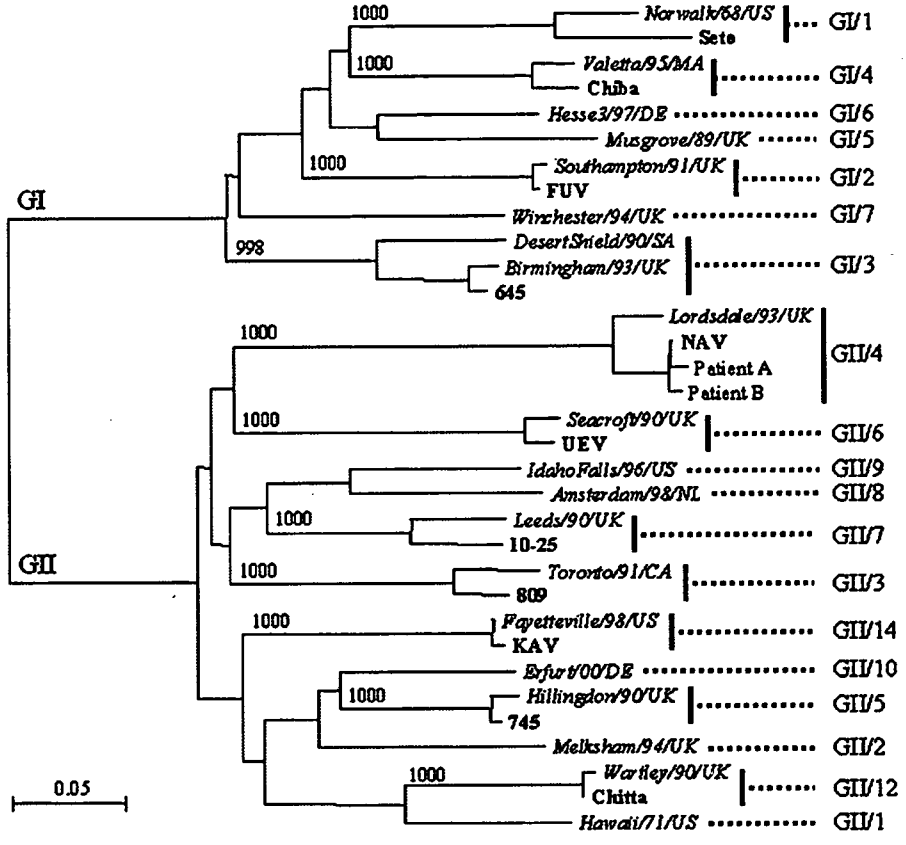

Fig. 1. Phylogenetic tree based on the capsid N/S domain region (GI, 294 nt; GII, 282 nt) constructed using neighbor-joining method. NoV strains for VLP and reference strains are represented in boldface and italics, respectively. Bar indicates the number of substitutions per site. The numbers on each branch indicate the bootstrap value of clusters including NoV strains for VLP. GenBank accession numbers for reference strains used in this analysis are as follows: Amsterdam/98/NL, AF195848; Birmingham/93/UK, AJ277612; DesertShield/90/SA, U04469; Erfurt/00/DE, AF427118; Fayetteville/98/US, AY113106; Hawaii/71/US, U07611; Hesse3/97/DE, AF093797; Hillingdon/90/UK, AJ277607; IdahoFalls/96/US, AY054299; Leeds/90/UK, AJ277608; Lordsdale/93/UK, X86557; Melksham/94/UK, X81879; Musgrove/89/UK, AJ277614; Norwalk/68/US, M87661; Seacroft/90/UK, AJ277620; Southampton/91/UK, L07418; Toronto/91/CA, U02030; Valetta/95/MA, AJ277616; Wartley/90/UK, AJ277618; Winchester/94/UK, AJ277809.

IgA antibodies specific to rNAV were detected from the 18th day post-onset. This specific fecal IgA seems to be followed by the reduction of PCR product or virus excretion, suggesting that fecal IgA was effective in the excretion of NoV.

The cross-reactivity of IgG in this study showed lower reaction to the heterologous type within the genogroup. Many serological studies in adults or children also demonstrated cross-reactivity within and between the genogroups [Treanor et al., 1993; Noel et al., 1997; Hale et al., 1998; Smit et al., 1999; Farkas et al., 2003; Rockx et al., 2005a]. Some reports showed that cross-reactivity was stronger or limited to the genogroups [Noel et al., 1997; Hale et al., 1998; Farkas et al., 2003]. In antibody ELISA using VLPs and their hyperimmune serums, low levels of cross-reaction were observed [Kamata et al., 2005; Hansman et al., 2006]. These findings indicated that NoV strains had a common epitope to stimulate the production of IgG. The common epitopes within and between the genogroups were previously described [Kitamoto et al., 2002; Yoda et al., 2003; Parker et al., 2005]. Yoda et al. [2003] described that the common epitope between genogroups may be located in the *N*-terminus of capsid protein. This region is highly conserved and located in the inner part of capsid protein surrounding the RNA genome [Prasad et al., 1999]. From the above results, cross-reactivity between genogroups may be difficult to produce.

As described in previous reports [Parker et al., 1994a, 1995; Smit et al., 1999], antibodies to NoVs were acquired at a young age and had cross-reactivity; therefore, it is unclear whether cross-reactive antibodies occurred after single infection, or after multiple infections with different genotypes of NoV. Our study demonstrated that cross-reactive antibodies to heterologous types were produced after primary single infection. We could observe that the production of IgG to the heterologous type was later and lower than the homologous type, and had a time lag in patient A. The time lag of IgG production may be related to antigenic differences among these GII NoV strains. The seroconverted against r809 of IgG and IgA in a serum of patient A at 2 years and 10 months are uncertain because the serum was obtained too long after the last serum sample was negative.

TABLE III. Detection of Serum IgM, IgA, and Fecal IgA to the 11 VLPs in Infantile Patients by ELISA

Patient (age)	Time post-onset of illness	RT-PCR[a]	Reciprocal of serum dilution					Existence of specific fecal IgA[a]	
			IgM		IgA				
			rNAV (GII/4)	Other VLPs	r809 (GII/3)	rNAV (GII/4)	Other VLPs	rNAV (GII/4)	Other VLPs
A (2 years)	5th day	+	**<50**	<50	<50	**<50**	<50	−	−
	12th day	+	**1,600**	<50	<50	**50**	<50	−	−
	18th day	+	**1,600**	<50	<50	**1,600**	<50	+	−
	33rd day	+w	**200**	<50	<50	**800**	<50	**NT**	NT
	40th day	−	**200**	<50	<50	**1,600**	<50	+	−
	60th day	−	**<50**	<50	<50	**50**	<50	+	−
	2 years 10 months	NT	**<50**	<50	50	**100**	<50	**NT**	NT
B (1 year)	2nd day	+	**<50**	<50	<50	**<50**	<50	**NT**	NT
	105th day	NT	**<50**	<50	<50	**400**	<50	**NT**	NT

Bold: homologous type.
[a]+, Positive; +w, positive but weak band; −, negative; NT, not tested.

A serum sample from patient A at 2 years and 10 months had IgG antibodies to the homologous type with a high titer and the heterologous type within the genogroup with a low titer. The existence of IgG antibodies to VLPs may have been maintained without re-infection or emerged from re-infection. Patient A might have been re-infected with GII/4 NoV or other GII NoV excluding these seven genotypes in GII, because the IgG titer to the heterologous type was ≥twofold higher and IgM responses were not observed. The data of IgG analysis suggested that the cross-reactivity of IgG is produced in the primary infection and then the level of IgG against NoVs will be raised each time NoV infection occurs throughout life.

IgM and IgA antibody responses were specific to the homologous genotype. Previous studies using adult sera also suggested that serum IgM and IgA antibodies might be more specific for the homologous type than the heterologous type [Parker and Cubitt, 1994b; Brinker et al., 1998, 1999; Hale et al., 1998; Rockx et al., 2005a; Tsugawa et al., 2006]. To interpret the cross-reactivity of IgM and IgA, further investigations are needed based on cases of NoV primary infection.

Recent studies have shown that HBGA may function as receptors of NoV through outbreak investigation [Hennessy et al., 2003; Rockx et al., 2005b] and volunteer challenge studies [Hutson et al., 2002; Lindesmith et al., 2003]. The relation between HBGA and immune response to NoV infection was unknown, because the HBGA type of these two patients was unknown, but these are cases, which were naturally infected with NoV and the typical symptoms appeared, and will represent general immune response to primary NoV infection. This is the first study on humoral immune response in putative primary NoV infection using 11 different antigenic and genetic types of VLPs. Our data will improve understanding of the humoral immune response to NoV infection. However, only two cases were examined in this study. To interpret the immunity for NoV infection, further investigations at the human level are needed.

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