Molecular Epidemiological Studies on Group C Rotavirus Infection

(C群ロタウイルス感染症に関する分子疫学的研究)

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CONTENTS

PREFACE

Rotaviruses are member of the *Reoviridae* family and are the major viral agents of diarrheal disease of animals and humans. The genome of rotaviruses consists of 11 segments of double-stranded RNA (dsRNA) enclosed in a double-shelled particle. Until recently, it was believed that there was only one rotavirus group and that all rotaviruses shared a common group specific antigen. However, a number of viruses which are morphologically similar to rotaviruses but do not share the group specific antigen and usual genome profile generally associated with ordinary rotaviruses have been identified (31, 32). These distinct rotaviruses, atypical rotaviruses, are now classified into six groups (groups B, C, D, E, F and G), whereas the ordinary rotaviruses are termed group A rotaviruses (36). Only three of these groups (A, B and C) are known to infect humans (36).

Group C rotaviruses were first recognized as a causative agent of gastroenteritis in piglets (2, 38). Thereafter, rotaviruses with electropherotypes and antigenic properties similar to the porcine virus were identified in humans (4). During the last decade, human group C rotaviruses (CHRVs) have been associated with several outbreaks of acute diarrhea in Asia (25, 29, 33), Europe (3, 6) and South America (13). More recently, CHRVs have been detected from sporadic cases of diarrhea in the United States (20). On the other hand, preliminary serological surveys have shown that the percentage of sera from adults and children positive for group C rotavirus antibodies was 11 to 42% (9, 29, 36). Thus, CHRV is globally distributed and is thought to be one of emerging pathogens in humans.

CHRV infection in Japan was first reported by Oseto et. al. (30) in 1985. Ushijima et. al. (47) also detected CHRVs from fecal specimens collected in the Tokyo area in 1987.

Since then, some researchers have recognized CHRV infections in individual locations (12, 27, 29). However, none of these investigations have carried out an epidemiological study that extends through several locations in Japan.

Detection of CHRV has usually been done by immune electron microscopy using reference antisera or by polyacrylamide gel electrophoresis of viral RNA, because this virus is not successfully propagated *in vitro*. Since these methods are complicated and time-consuming, simple and rapid diagnostic procedures may be valuable for the timely construction of appropriate infection control measures in hospitals and for the gathering of epidemiological data to establish the importance of CHRV in human diarrheal diseases.

Genome electropherotyping is widely applied to epidemiological studies on group A rotavirus infection, and various genome electropherotypes have been recognized to date (43). Although some investigators reported that RNA profiles of CHRV isolates were different from those of porcine group C rotavirus isolates (4, 33), little is known about the variation of genome electropherotypes among the CHRVs.

In group A rotavirus, fourteen serotypes have been recognized on the basis of cross neutralization test with hyperimmune sera (5, 10, 43). The main serotype-specific neutralization antigens have been demonstrated to be located on VP7 which is encoded by genome segment 7, 8 or 9 (43). Comparative genetic analysis of the VP7 genes revealed a high degree of sequence homology among group A rotaviruses belonging to the same serotype, while a high degree of sequence divergence among the different serotypes (14, 17). Therefore, serotypes of group A rotaviruses could also be differentiated by cross hybridization assays with cDNA probes of the VP7 genes (11) and the PCR method using serotype-specific primers (16). For group C rotavirus, an outer capsid glycoprotein corresponding in molecular mass to the VP7 protein of group A rotavirus was identified (22) and shown to be encoded by eighth genome segment (34). Sequence variation

between different animal and human group C rotavirus VP7 genes was demonstrated by cross hybridization studies (21). On the other hand, surprisingly high levels of sequence conservation of the VP7 gene among three CHRV strains were recently demonstrated by Grice et al. (18). However, knowledge of the genetic variation among CHRVs is still limited.

For the epidemiological study of CHRV infection, the author first developed reverse passive hemagglutination and latex agglutination tests using anti-CHRV monoclonal antibodies to detect CHRVs in fecal specimens. Subsequently, the genome electropherotypes and the VP7 gene sequences of CHRV clinical isolates, which were detected in various locations in Japan, were analyzed in order to investigate the genetic and antigenic divergence of CHRVs.

CHAPTER I

Rapid Detection of Human Group C Rotaviruses by Reverse Passive Hemagglutination and Latex Agglutination Tests using Monoclonal Antibodies

INTRODUCTION

Rotaviruses are the most common causative agent of gastroenteritis in infants and young children in many countries (23). Until recently, it was believed that there was only one rotavirus group and that all rotaviruses shared a common group specific antigen. However, a number of viruses which are morphologically similar to rotaviruses but do not share the group specific antigen and usual genome profile generally associated with ordinary rotaviruses have been identified (31, 32). These distinct rotaviruses, atypical rotaviruses, are now classified into six groups (groups B, C, D, E, F and G), whereas the ordinary rotaviruses are termed group A rotaviruses (36).

Group C rotaviruses were first identified as a cause of gastroenteritis in pigs (2, 38). Thereafter, rotaviruses with electropherotypes and antigenic properties similar to the porcine virus were identified in humans (4). Recently, a large-scale outbreak of diarrhea caused by human group C rotavirus (CHRV) in school children in Japan has been reported (25), and others have also observed additional CHRV infections (3, 6, 30, 33, 47). On the other hand, preliminary serological surveys have shown that the percentage of sera from adults and children positive for group C rotavirus antibodies was 11 to 42% (9, 29, 36). These results suggest that CHRV may be emerging enteric pathogens in humans.

Detection of CHRV has usually been done by immune electron microscopy using reference antisera or by polyacrylamide gel electrophoresis of viral RNA (RNA-PAGE), because CHRV is noncultivatable. Since these methods are complicated and time-consuming, simple and rapid diagnostic procedures may be valuable for the timely construction of appropriate infection control measures in hospitals and for the gathering of epidemiological data to establish the importance of CHRV in human diarrheal diseases. In this chapter, I developed reverse passive hemagglutination (RPHA) and latex

agglutination (Lx-Ag) tests using anti-CHRV monoclonal antibodies (MAbs) for the rapid detection of CHRV.

MATERIALS AND METHODS

Viruses

Five CHRV isolates antigenically identified by reference sera were obtained from Dr. M. Oseto, Ehime Prefectural Institute of Public Health and Dr. K. Matsumoto, Fukui Prefectural Institute of Public Health. Forty-six fecal specimens obtained from patients with acute diarrhea in Okayama, as well as the clinical isolates, had been examined for the presence of rotaviruses by electron microscopy and RNA-PAGE. Ten percent suspensions of the fecal specimens in phosphate buffered saline (PBS, pH 7.2) were homogenized with a vortex mixer, and were centrifuged at 2,000 X g for 10 min. The supernatants, which were referred to as fecal extracts, were subjected to the assays. The Wa strain of human group A rotavirus (AHRV) was propagated in MA104 cells, and the culture supernatant was used for the assays.

MAbs

Two selected MAbs were used in this chapter. MAb 5A12, whose subclass was immunoglobulin G2b, was described previously (12). MAb 13A3, whose subclass was immunoglobulin G1, was newly established in this study. The methods used for production and selection of hybridomas have been described elsewhere (12). Briefly, splenocytes of Balb/c mice immunized with purified CHRV were fused with murine myeloma XAg63 cells with polyethylene glycol 4000. Hybridomas were screened for the production of anti-CHRV antibodies by an enzyme-linked immunosorbent assay (ELISA) using the purified virion. Positive cultures were subcloned twice by limiting dilution method. MAbs were purified from mouse ascitic fluids by affinity chromatography.

Characterization of MAbs

The avidity of MAbs to CHRV was checked by the ELISA system (12) by using

homologous MAbs as a capture antibody and a detector antibody. Briefly, wells of a microtiter plate were coated with 100 μl of each MAb solution (10 μg/ml). Purified CHRV was added to each wells, and the plate was incubated at 37°C for 1 h. After washing, 100 μl of biotin-conjugated MAb (0.5 μg/ml) which was identical with the capture antibody was added and then horseradish peroxidase-conjugated streptavidin solution was dispensed into the wells. Finally, the substrate solution (3,3',5,5'-tetramethlybenzidine in citrate buffer) was added to each well, and the color was measured by a spectrophotometer at wavelength of 450 nm.

To determine whether MAbs reacted with the outer or inner capsid, the purified CHRV was treated with various concentrations of EDTA (2, 5 and 10 mM) for 15 min at room temperature. The treated virus was then tested by the ELISA system used for determination of the avidity of MAbs.

Preparation of SRBCs coated with MAb

A 2.5% suspension of sheep red blood cells (SRBCs) fixed with glutaraldehyde was incubated with an equal volume of 0.005% tannic acid solution for 15 min at 37°C. The tanned SRBC suspension was mixed with an equal volume of anti-CHRV MAbs in 0.15 M phosphate buffer (pH 6.4), and the mixture was incubated at 37°C for 15 min. The MAb-coated SRBCs were suspended in PBS containing 1% heat-inactivated normal rabbit serum. SRBCs coated with normal mouse immunoglobulin G in the same manner were used as the control.

RPHA tests

To eliminate nonspecific reactions, fecal extracts were preincubated with SRBCs fixed with glutaraldehyde at 37°C for 1 h. After centrifugation, the supernatants were subjected to the RPHA tests by using V-shaped microtiter trays. Serial twofold dilutions of the samples in 25 μl of normal rabbit serum-PBS were made in duplicate. In one

dilution series, 25 μl of a 0.7% suspension of the MAb-coated SRBCs was added to each well. In the other series, the same amount of control SRBC was added. After shaking, the tray was allowed to stand at room temperature. Hemagglutination patterns were observed after 1 h. The reciprocals of the endpoint dilutions of hemagglutination with MAb-coated SRBCs or control SRBCs were referred to as RPHA titers. The RPHA index was then defined as follows; RPHA index =RPHA titer with MAb-coated SRBCs / RPHA titer with control SRBCs. If the RPHA titer was <4, it was regarded as 2. An RPHA index of ≥4 was judged to be CHRV positive.

Preparation of MAb-coated Latex particles

Latex particles (Lx) (Takeda Co. Ltd.) were coated with MAb 13A3 according to the manufacturer's instructions. Briefly, a 0.5% suspension of Lx in 0.1 M Tris-HCl, pH 8.0 (TB) was incubated with an equal volume of the MAb in TB at 37°C for 1 h with continuous shaking. The MAb-coated Lx (MLx) were washed with TB and resuspended in TB containing 1% normal rabbit serum. The 1% suspension of MLx thus made was kept at 4°C until use. Lx coated with normal mouse immunoglobulin G were used as the control.

An Lx-Ag test

Two aliquots of 10 μl of fecal extracts were placed on a slide, and equal volumes of MLx or control Lx were added just beside each drop. The drops were carefully mixed with a plastic rod. Then the slide was gently tilted by hand during the reaction time. After 2 min, agglutination was macroscopically observed. The samples which developed agglutination with MLx but not with control Lx were judged to be CHRV positive. When the samples developed non-specific agglutination, they were tested again after treatment with trichlorotrifluoroethane (Daiflon S-3, Daikin Co. Ltd.).

Sensitivity of RPHA and Lx-Ag tests

The sensitivity of RPHA and Lx-Ag tests was determined as described by Sanekata et al. (40). The number of virus particles in the purified virus solution of CHRV was counted by electron microscopy, with Lx as a reference, and the serial two-fold dilutions of the solution were examined by these methods.

RESULTS

Characterization of MAbs

The abidity of the MAbs to CHRV was checked by the ELISA system (12). The reactivity of MAb 13A3 (optical density; 0.782) was slightly superior to that of MAb 5A12 (optical density; 0.567).

The antigenic sites recognized by the MAbs were determined by ELISA reactivity with double- or single-shelled virus particles. The reactivity of MAb 5A12 was decreased after EDTA treatment of the virus, whereas that of MAb 13A3 remained at a constant level or increased (Fig. 1). These results suggest that MAb 5A12 and 13A3 recognize the outer and inner capsids of CHRV, respectively. To verify this conclusion, immune electron microscopy with single-shelled virus was performed. The EDTA-treated CHRV solution (25-μl volumes) was mixed with an equal volume of each MAb solution (1 μg/ml). Drops of the reaction mixture were placed on grids, stained with uranyl acetate. As a result, MAb 13A3 strongly agglutinated the single-shelled viruses (Fig. 2), whereas MAb 5A12 failed to agglutinate them (data not shown).

Detection of CHRV by RPHA tests

To optimize the condition for preparation of sensitized erythrocytes, SRBCs coated with either of the MAbs at various concentrations were reacted with serial two-fold dilutions of partially purified fecal specimens containing CHRV (Table 1). For both MAbs, the RPHA titer reached a plateau at a concentration of 80 μg/ml. SRBCs coated with MAb 5A12 (RBC5A12) and with MAb 13A3 (RBC13A3) at this concentration were used thereafter.

Five clinical isolates which had been identified as CHRV with reference antisera and the Wa strain of AHRV were first tested to check the specificity of these tests (Fig. 3).

Four of the five CHRV isolates were positive by the RPHA test with RBC5A12, whereas all of them were positive by that with RBC13A3. The Wa strain was not react with either MAb-coated SRBCs. The specificity of the tests was also confirmed by a blocking test. The CHRV isolates were incubated with each MAb solution (100 μg/ml) for 1 h at room temperature and then the mixture was tested by the RPHA tests. With either MAb-coated SRBCs, the RPHA titers of the samples drastically reduced by pretreatment with homologous MAbs.

To evaluate the practical usefulness of the RPHA tests, fecal specimens obtained from patients with acute diarrhea were examined by the tests and the results were compared with those determined by RNA-PAGE (Fig. 3). The RPHA titers with RBC13A3 were generally higher than those with RBC5A12. There was concordance between the RPHA test with RBC5A12 and RNA-PAGE in 44 (95.6%) of 46 samples, while the diagnoses by the RPHA test with RBC13A3 were in complete agreement with those by RNA-PAGE.

Detection of CHRV by an Lx-Ag test

To further simplify the detection of CHRV, the Lx-Ag test with MAb 13A3 was developed. The optimum condition in which to prepare MLx was studied by the same manner used in the RPHA system. The concentration of MAb showing the most sensitive detection was found to be 1,200 μg/ml (Table 2).

Table 3 shows the CHRV detection from 5 clinical isolates and 46 fecal specimens by the Lx-Ag test. Specific agglutination was observed within 30 s in 21 out of 22 specimens known to contain CHRV by either reference sera or RNA-PAGE. Nonspecific agglutinin in a CHRV sample and an AHRV sample could not be eliminated even after treatment of these samples with trichlorotrifluoroethane.

Sensitivity of RPHA and Lx-Ag tests

The minimum numbers of CHRV detectable by the RPHA tests with RBC5A12 and

RBC13A3 were 5.5 x 10^7 and 1.5 x 10^7 particles per ml, respectively. On the other hand, 3.0 x 10^8 particles per ml were detectable by the Lx-Ag test.

DISCUSSION

In this chapter, I employed RPHA and Lx-Ag tests, which had been widely used in AHRV detection (19, 40, 41), for the specific detection of CHRV. These tests have several obvious advantages over the ELISA established previously (12), although the ELISA is simple and sensitive as compared with immune electron microscopy or RNA-PAGE. The first advantage is detection time. It takes only 2 min to detect CHRV by the Lx-Ag test and 3 h by the RPHA tests, whereas it takes at least 2 days by the ELISA. The second advantage is the simplicity of the test systems. Once SRBCs and Lx coated with MAb are ready, the reactions involve a single step with these methods. Furthermore, no special equipment is required for the Lx-Ag test and the only equipment required for the RPHA tests is a microtitration set. Consequently, because the present procedures can be used in small clinical laboratories which are not equipped well, they would be valuable for epidemiological studies to define the distribution of CHRV in the world and to establish its importance in human diarrheal disease. Although the sensitivity of these tests is not comparable with that of the ELISA, it is sufficient for routine diagnostic requirements in clinical settings.

In case of the application of RPHA and Lx-Ag for CHRV detection, the most important issue was the selection of antibodies. The possible sources of group C rotavirus antigens for the preparation of polyclonal antibodies are cultivatable animal group C rotaviruses, such as the Cowden strain (37) and the Shintoku strain (46). In the detection of AHRV by the immunological methods, however, it has been reported that antibodies raised against human rotavirus are superior to ones raised against other animal rotaviruses (8, 39, 49, 50). In fact, the antigenic diversity between human and animal group C rotaviruses has been demonstrated on the bases of their reactivity to antisera in immune electron

microscopy analysis (4, 33). Recently, the genetic diversity among group C rotavirus has also been reported (21, 35). Because no CHRVs have been successfully propagated in vitro, it is difficult to constantly prepare polyclonal antibodies against CHRVs. Therefore I used MAbs raised against CHRV, once they were established; the supply is unlimited and the quality control of diagnostic systems is easy.

The key diagnostic reagent in the present treatise was MAb 13A3, which recognized the inner capsid of CHRV, because the RPHA test with MR13A3 provided us with more sensitive detection of CHRV and the Lx-Ag test using MAb 13A3 successfully detected CHRV within 2 min. Furthermore, my preliminary data suggest that MAb 13A3 cross-react with both bovine and porcine group C rotavirus (data not shown). If testing of additional isolates from animals confirms this cross-reactivity, MAb 13A3 could be useful in monitoring the postulated transmission of group C rotavirus between humans and animals. Although the RPHA and Lx-Ag tests using MAb 13A3 are promising diagnostic methods, antigenic variation in the epitope on the inner capsid must be taken into consideration. Group A rotaviruses have been classified into subgroups 1 and 2 by the antigenicity of the inner capsid protein (VP6) (23, 24). Cooke et al. (7) have reported that the major inner capsid protein sequence alignments revealed a region of 10 amino acids which were significantly different between human and porcine group C rotaviruses. More specimens must be examined before the present procedures can be evaluated conclusively.

In conclusion, the RPHA and Lx-Ag tests were demonstrated to be simple and rapid diagnostic procedures for CHRV infection. The Lx-Ag test is recommended as a screening test, and the RPHA tests are suitable for more precise and quantitative determination.

SUMMARY

Reverse passive hemagglutination (RPHA) tests and a latex agglutination test using monoclonal antibodies (MAbs) were developed for the rapid detection of noncultivatable CHRVs. For RPHA tests, two MAbs, MAb 5A12 recognizing the outer capsid and MAb 13A3 recognizing the inner capsid, were separately used for the coating of sheep red blood cells (SRBCs). Forty-six fecal specimens were examined to confirm the practical usefulness of the tests. As a result, there was concordance between the RPHA test using SRBCs coated with MAb 5A12 and polyacrylamide gel electrophoresis of viral RNA (RNA-PAGE) in 44 (95.6%) of 46 samples, while the diagnoses by the RPHA test using SRBCs coated with MAb 13A3 were in complete agreement with those by RNA-PAGE. Furthermore, a latex agglutination test with MAb 13A3 was also developed, and this test was fast enough and sensitive enough to successfully detect the viruses from most fecal specimens within 2 min. The present procedures would be useful for the diagnosis of CHRV infections in clinical laboratories which are not equipped well.

TABLE 1. Optimum concentration of MAbs for the preparation of MAb-coated SRBCs

MAbs	Concentration of MAbs (μg/ml)	RPHA titer of fecal specimens			
		538	I57	462	PBS
5A12	320	80	160	80	<10
	160	80	160	40	<10
	80	80	160	40	<10
	40	20	80	20	<10
	0	<10	<10	<10	<10
13A3	320	1,280	2,560	320	<10
	160	2,560	1,280	320	<10
	80	2,560	1,280	320	<10
	40	1,280	1,280	320	<10
	0	<10	<10	<10	<10

TABLE 2. Optimum concentration of MAb 13A3 for the preparation of MLx

Sample	Concentration of MAb 13A3 (μg/ml)	Sample diluton								
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
538	2,400	+++[a]	+++	+++	+++	+++	+++	+++	−	−
	1,200	+++	+++	+++	+++	+++	+++	+++	−	−
	600	+++	+++	+++	+++	+++	++	+	−	−
	300	+++	+++	+++	+++	++	+	−	−	−
I57	2,400	+++	+++	+++	+++	+++	−	−	−	−
	1,200	+++	+++	+++	+++	++	+	−	−	−
	600	+++	+++	+++	−	−	−	−	−	−
	300	+++	++	+	−	−	−	−	−	−
462	2,400	++	+	−	−	−	−	−	−	−
	1,200	++	+	−	−	−	−	−	−	−
	600	++	−	−	−	−	−	−	−	−
	300	+	+	−	−	−	−	−	−	−

[a]Agglutination patterns were classified as follows;
+++ : Agglutination was observed within 30 s.
++ : Agglutination was observed from 30 s to 1 min.
+ : Agglutination was observed from 1 min to 2 min.
− : No agglutination was observed.

TABLE 3. Detection of rotaviruses by the Lx-Ag test

Rotavirus detected by RNA-PAGE (no. of samples)	No. of Lx-Ag test results		
	Positive	Negative	ND[a]
CHRV (22)[b]	21	0	1
AHRV (9)	0	8	1
None (20)	0	20	0

[a]ND, could not be determined because of nonspecific agglutination.
[b]Five clinical isolates and 17 fecal specimens.

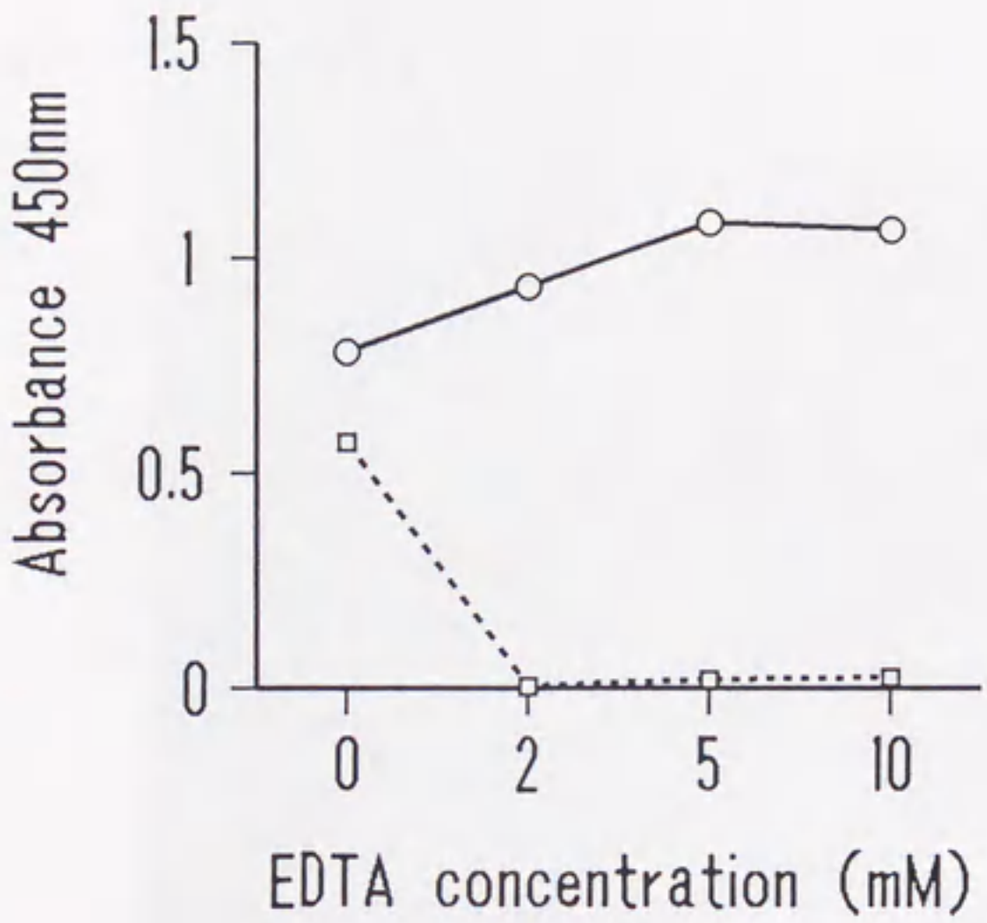

FIG. 1. Effect of EDTA treatment on MAbs binding. Purified CHRV was treated with various concentrations of EDTA for 15 min at room temperature. The EDTA-treated virus was tested for the reactivity in an ELISA by using same MAb as a capture antibody and a detector antibody. Symbols: (○) MAb 13A3; (□) MAb 5A12.

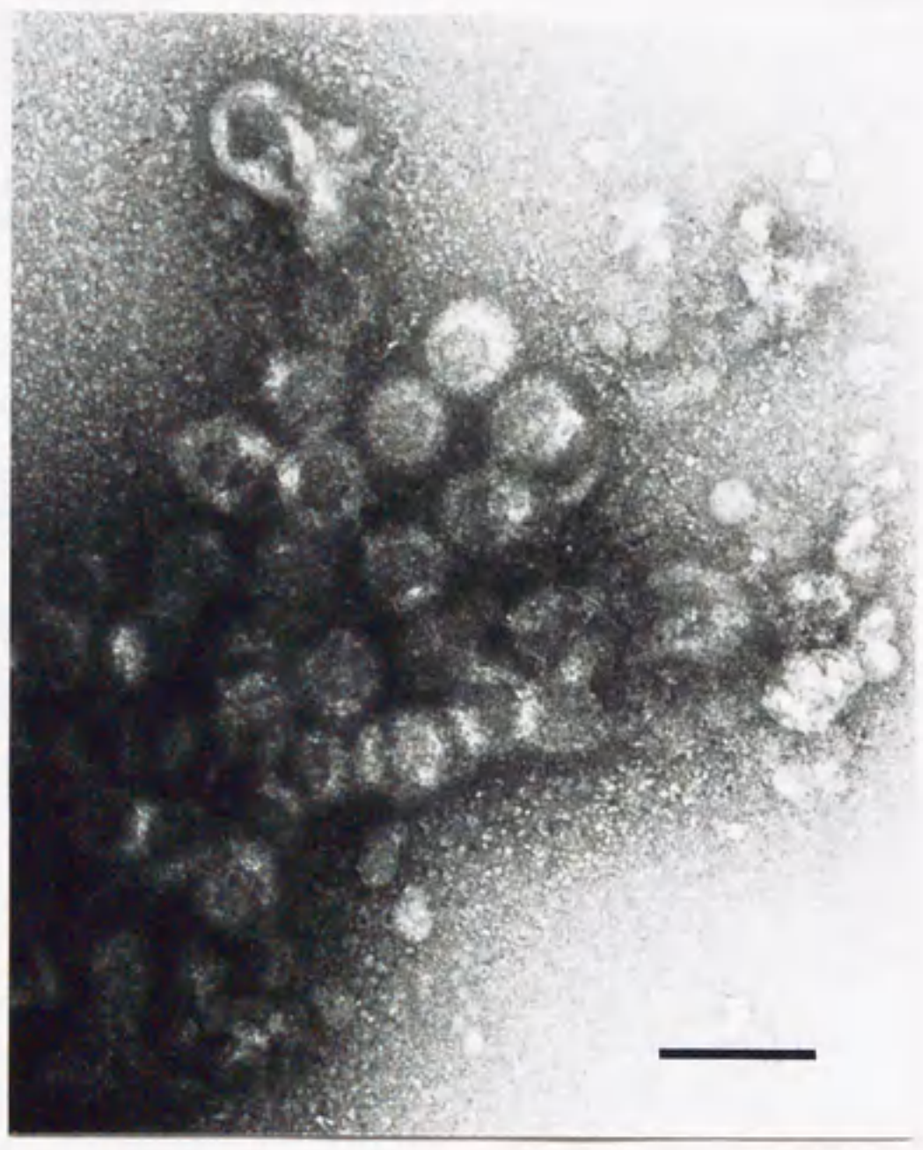

FIG. 2. Immune electron micrograph of EDTA-treated (single-shelled) CHRV incubated with MAb 13A3. Barr, 100 nm.

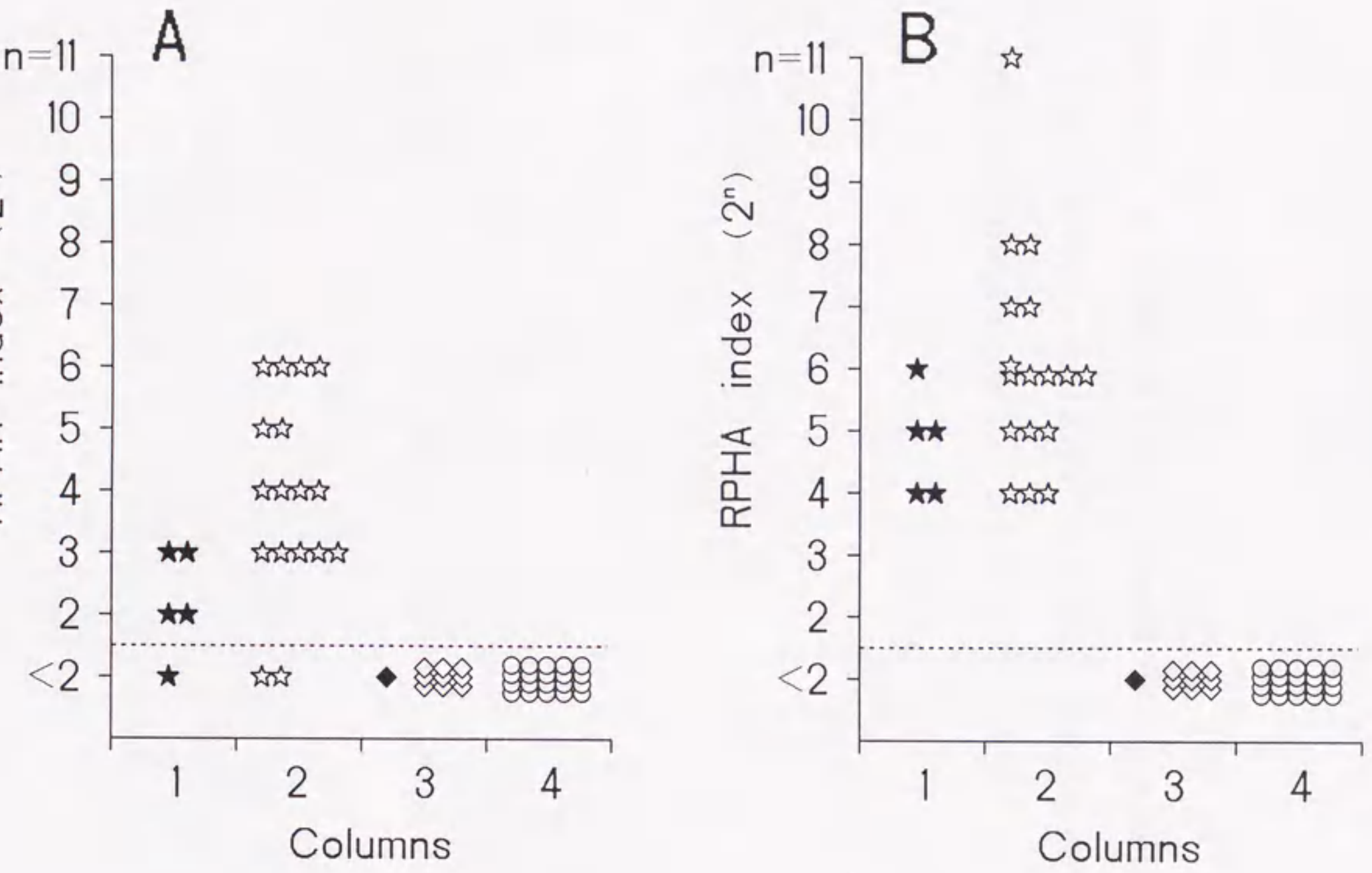

FIG. 3. RPHA indices of the clinical isolates and fecal specimens with RBC5A12 (A) and RBC13A3 (B). Column 1, 5 clinical isolates (★) of CHRV identified with reference antisera; column 2, 17 fecal specimens (☆) known to contain CHRV; column 3, AHRV samples (◆, Wa strain; ◇, 9 fecal specimens containing AHRV); column 4, 20 fecal specimens (○) in which no rotavirus was detectable. Isolates and fecal specimens with an RPHA index of four (2^2) or more were considered as CHRV positive.

CHAPTER II

Molecular Analysis of Outer Capsid Glycoprotein (VP7) Genes from Two Isolates of Human Group C Rotavirus with Different Genome Electropherotypes

INTRODUCTION

Rotaviruses are member of the *Reoviridae* family and have a genome that consists of 11 segments of dsRNA. On the basis of antigenic cross-reactivity and the relative electrophoretic mobilities of their dsRNA segments, rotaviruses are divided into seven groups (groups A to G) (36). Only three of these groups (A, B and C) are known to infect humans (36). Human group A rotavirus is the most important cause of acute gastroenteritis in infant and young children (43). Human group B rotavirus have been associated with several outbreaks of diarrhea in adults in China (36). CHRV has been shown to cause both outbreaks and sporadic cases of diarrhea in many parts of the world (6, 9, 25, 33).

In group A rotavirus, 14 serotypes are recognized on the basis of cross-neutralization tests with hyperimmune sera (5, 10, 43). The major serotype-specific epitopes of group A rotavirus were demonstrated to be located in the major outer capsid glycoprotein VP7, which was encoded by genome segment 7, 8 or 9 (43). Although serological diversity within group C rotaviruses was suggested in a preliminary cross-neutralization study (45) , no serotyping has so far been done. Nevertheless, an outer capsid glycoprotein corresponding to the VP7 of group A rotavirus was identified in group C rotavirus (22) and was shown to be encoded by the eighth genome segment (34). Sequence variation of the VP7 gene was demonstrated between animal group C rotaviruses and CHRVs by cross-hybridization studies (21). In contrast, surprisingly high levels of sequence conservation of the VP7 gene among three CHRV strains were recently demonstrated by Grice et al. (18).

Fujii et al. (12) have recently demonstrated that genome electropherotypes of CHRVs isolated in Okayama, Japan were classified into two patterns (patterns I and II). In the

present chapter, I performed a comparative nucleotide and amino acid sequence analysis of the VP7 genes from two isolates with different electropherotypes in order to investigate the genetic and antigenic divergence of CHRVs.

MATERIALS AND METHODS

Viruses

Sources of 11 CHRV isolates used in this chapter are shown in Table 4. Most of the isolates were detected in or around Okayama City. The isolates were identified as CHRV by specific monoclonal antibodies in the chapter I. Two isolates (OK118 and OK450) were used as templates of cDNA cloning.

RNA extraction

Two hundred microliters of 20% fecal suspensions containing CHRV were extracted with an equal volume of trichlorotrifluoroethane, and the supernatants were adjusted to contain 10 mM EDTA, 0.6% sodium dodecyl sulfate (SDS) and 300 μg of proteinase K per ml. The suspensions were incubated for 1.5 h at 40℃ and then extracted with phenol-chloroform. Viral dsRNA was further purified with RNAID kit (BIO 101, Inc.) according to the manufacturer's instructions. Finally, the RNAs were resuspended in 20 μl of diethyl pyrocarbonate-treated water and stored at -30℃ until use.

Oligonucleotide primers

Oligonucleotide primers corresponding to the 5' end (G8S: 5'-GGCATTTAAAAAAGAAGAAGCTGT-3') and the 3' end (G8A: 5'-AGCCACATGATCTTGTTTACGC-3') of the published VP7 gene sequence (34) were synthesized and used for reverse transcription (RT)-PCR amplification of the VP7 gene. Two primers (G8SH, 5'-CCCAAGCTTGGCATTTAAAAAAGAAGAAGCTGT-3' and G8AB, 5'-CCCGGATCCAGCCACATGATCTTGTTTACGC-3') were also prepared for cDNA cloning of the full-length VP7 sequence. The underlined parts of these sequences were restriction sites introduced for cloning.

RT-PCR of the VP7 genes

Genomic dsRNA from the clinical isolates was dissolved in 15% dimethyl sulfoxide (DMSO) solution containing the primers (G8S and G8A), and heated at 97℃ for 5 min. After rapid cooling, the denatured RNA was converted to cDNA with RNaseH⁻ reverse transcriptase (Superscript; Life Technologies Inc.) at 42℃ for 60 min. The remaining RNA was hydrolyzed by addition of NaOH, followed by neutralizing with HCl and Tris-HCl (pH 8.0). The resultant cDNA was purified with a Suprec-02 column (Takara Shuzo Co., Ltd.) and amplified with *Taq* DNA polymerase for an initial 3-min incubation at 72℃, followed by 30 cycles of PCR (94℃ for 1 min, 48℃ for 2 min and 72℃ for 2 min) and a final 7-min incubation at 72℃.

Cloning and sequencing of the VP7 genes

The VP7 genes of two clinical isolates (OK118 and OK450 strains) were amplified by PCR using the G8SH and G8AB primers. The PCR products were digested with restriction endonucleases (BamHI and HindIII) and then cloned into plasmid pUC18. Five individual recombinants were isolated and both strands of the cloned DNA were sequenced by the dideoxynucleotide chain-termination method (Applied Biosystems).

Dot blot hybridization

The VP7 genes amplified from clinical isolates by RT-PCR were extracted with phenol-chloroform and precipitated with ethanol. The genes were dissolved in distilled water and purified with Suprec-02 columns. Equivalent amounts (200 ng) of the genes were denatured by boiling and blotted onto nylon membranes. Probes were generated by labeling the VP7 genes from the OK118 and OK450 strains with digoxigenin-11-dUTP (Boehringer) by the PCR method. Hybridization was performed at 52℃ for 16 h in hybridization buffer consisting of 50% formamide, 5X SSC (1X SSC contains 0.15 M NaCl and 0.015 M sodium citrate [pH 7.0]), 7% SDS, 2% blocking reagent (Boehringer), 50 mM sodium-phosphate (pH 7.0), 0.1% N-lauroylsarcosine, 50 μg of denatured salmon

sperm DNA per ml and 10 ng of the DIG-labeled probe per ml. After hybridization, the membranes were washed twice in 0.1X SSC containing 0.1% SDS at 68℃ for 15 min. Colorimetric detection of bound probe was carried out as recommended by the manufacturer.

Sequence data analysis

Nucleotide sequence data were analyzed by the GENETYX-MAC version 6.0 program. The program MAlign, version 1.0, was also used for sequence alignments.

Accession numbers

The nucleotide sequence data of the VP7 genes from the OK118 and OK450 strains have been submitted to the DDBJ DNA database and have been assigned the accession numbers D87543 and D87544, respectively.

RESULTS

Observation of two different genome electropherotypes in an epidemic in Okayama Prefecture

Electropherotyping of 11 clinical isolates revealed two distinct genome electropherotypes, patterns I and II (Table 4). The OK118 strain was selected as a representative of 6 isolates with pattern I, while the OK450 strain was selected as a representative of 5 isolates with pattern II. RNA profiles of the OK118 and OK450 strain are shown in Fig. 4. Although both strains exhibited the typical 4-3-2-2 profile of group C rotavirus, the 5th, 7th and 10th genome segments of the OK450 strain migrated slowly as compared with those of the OK118 strain; other minor differences were observed in the 3rd, 4th and 8th segments.

Nucleotide and deduced amino acid sequences of the VP7 gene from the OK118 and OK450 strains

A sequence comparison of the VP7 genes from the OK118 and OK450 strains is shown in Fig. 5. Both sequences were 1,063 nucleotides in length and contained a single open reading frame beginning at the 49th nucleotide from the 5' end and terminating 16 bases upstream from the 3' end. The open reading frame consisted of 999 nucleotides and encoded 332 amino acids. There were 46 nucleotide substitutions between the two sequences and these substitutions were scattered throughout the gene. The overall nucleotide identity value was 95.7%.

The VP7 genes of the OK118 and OK450 strains were then compared with the published sequences of four CHRV strains. As shown in Table 5, a surprising level of sequence conservation was observed between the OK118 strain and a Japanese isolate (88-220 strain). The gene sequence of the OK118 strain was also similar to those of foreign

isolates (Bristol, Preston and Belém strains), with overall identities of 98.0% to 98.2%, whereas the overall nucleotide identities between the OK450 strain and the other strains were relatively low (from 95.3% to 95.7%).

Figure 6 shows the alignment of the deduced amino acid sequences of VP7 from the OK118 and OK450 strain with the published sequences from four CHRV strains. The amino acid identity values among six strains are summarized in Table 5. The overall identity between the OK118 and OK450 sequences was 96.7%. Although there were 11 amino acid substitutions between the two sequences, no clustered amino acid divergent region was observed. There were three conserved sites that were potential N-glycosylation sites (Asn-X-Ser/Thr) in both VP7 polypeptides. Among five of the six strains (all except the OK450 strain), high degrees of sequence identity (more than 98.4%) were observed, while there were around 96% identities between the OK450 strain and the other strains. In addition, unique amino acid substitutions occurred at nine positions in the OK450 sequence.

Dot blot hybridization analysis of the VP7 genes from the clinical isolates with different electropherotypes using the OK118 and OK450 cDNA probes

Dot blot hybridization analysis was performed to evaluate the existence of genome divergence represented by the OK118 and OK450 strains in nine clinical isolates. First, various hybridization conditions were examined to differentiate the VP7 gene of the OK118 strain from that of the OK450 strain. As a result, under highly stringent conditions (50% formamide, 5X SSC and 52℃), each probe could strongly hybridize with the gene of homologous strains and weakly reacted with that of heterogous strains.

Amplified VP7 cDNAs from 11 isolates, including the prototypes, were spotted on nylon membranes and hybridized with the labeled probe under the same high-stringency conditions. As shown in Fig. 7, the OK118 probe produced strong hybridization signals

with the corresponding genes of all pattern I isolates and one pattern II isolate (OK595 strain), and weak signals were observed with those of other isolates. In contrast, the OK450 probe strongly reacted only with the genes of four pattern II isolates. The dot blot results of 11 clinical isolates are summarized in Table 4.

DISCUSSION

Genome electropherotyping is widely applied to epidemiological studies of group A rotavirus infections, and various genome electropherotypes have been recognized to date (43). Although some investigators reported that RNA profiles of CHRV isolates were different from those of porcine group C rotavirus isolates (4, 33), little is known about the variation of genome electropherotypes among the CHRVs. I showed that genome electropherotypes of CHRVs isolated in an epidemic in Okayama Prefecture during 1988 to 1990 were classified into patterns I and II, and that dominant patterns changed from year to year.

In this chapter, I demonstrated that electropherotypes of CHRVs were related to variations in the outer capsid glycoprotein VP7 gene. The OK118 and OK450 strains were selected as prototypes of patterns I and II, respectively, and a comparative nucleotide and amino acid sequence analysis of the VP7 gene of these strains was performed. Although the sequences were identical in size and both contained single open reading frames with the same size, there were 46 nucleotide substitutions between them. The comparison of the predicted VP7 sequence also showed that amino acid substitutions occurred at 11 positions. These data indicate that the OK118 and OK450 strains are genetically and perhaps antigenically distinct.

The dot blot analysis revealed almost exclusive cross-hybridization of the OK118 and OK450 cDNA probes to the clinical isolates with patterns I and II, respectively, suggesting that relative sequence diversity in the VP7 gene exists between the two electropherotypes. However, an exception was the OK595 strain, which exhibited the pattern II electropherotype, but possessed the OK118-type VP7 gene. Nakagomi et al. (26) and Ward et al. (48) have described naturally occurring reassortants between group A

rotaviruses belonging to different genogroups. It is therefore of interest to know whether the OK595 strain is a reassortant between patterns I and II.

I compared the VP7 gene sequences of the OK118 and OK450 strains with published sequences of VP7 genes from four CHRV strains. The VP7 gene of the OK118 strain exhibited very high levels of homology with the genes of all four strains. In contrast, the overall nucleotide identities between the OK450 strain and the other strains were from 95.3% to 95.7%, and 35 nucleotide substitutions, nine of which were predicted to give amino acid changes, were uniquely observed in the OK450 sequence, indicating that the OK450 strain had a distinct VP7. It has been proposed that CHRV was originally derived from pigs through interspecies transmission (33). The VP7 genes of the OK450 and OK118 strains were then aligned with the corresponding gene of a porcine group C rotavirus (Cowden strain). The nucleotide identity value between the OK450 strain and the Cowden strain (83.7%) was similar to the value between the OK118 strain and the Cowden strain (84.1%). These data imply that the OK450 strain is not an intermediate strain between porcine and human viruses. Tsunemitsu et al. (44) have recently described the sequence comparison of the VP7 gene among human, porcine and bovine group C rotaviruses. Although the Cowden strain was most closely related to a human strain as compared with other strains, there was a limited (81.9%) homology between Cowden and the human strains. Additional sequence analyses of the VP7 genes from group C rotaviruses isolated from humans, pigs and other species are needed to yield insights into the evolutionary origin of CHRV.

In group A rotavirus, isolates belonging to the same serotype are highly homologous (more than 90%) in the VP7 sequence (17), while isolates in different serotypes show significant VP7 divergence, and several discrete regions of variable amino acid sequence are defined (14). It is thought that these variable regions are involved in the

determination of serotype specificity. Among six CHRV strains, sequence heterogeneity of the VP7 protein was relatively small (less than 4%) in comparison with that of the group A rotavirus, and no clustered amino acid variable region was observed. It seems likely that these strains belong to the same group C rotavirus serotype. However, further studies using serologic assays are required to define whether serotypes exist within CHRVs, because the minor amino acid changes may affect the antigenic properties of the VP7 protein.

In conclusion, I have demonstrated that CHRVs with two different electrophererotypes were prevalent in Okayama Prefecture and also have shown that the VP7 gene of CHRV varied in accordance with the changes in its genome electropherotypes. Production of monoclonal antibodies which differentiate the OK118 strain from the OK450 strain is required to clarify the antigenic relationship between the two different electropherotypes.

SUMMARY

Nucleotide sequences of the VP7 gene of CHRV were determined for two strains isolated in Okayama, Japan during a 1988 to 1990 epidemic. These strains, OK118 and OK450, were selected as prototypes of two different electropherotypes, patterns I and II, respectively. The genes were identical in size (1,063 bp) and both contained single open reading frames encoding 332 amino acids. The alignment of two sequences revealed 46 nucleotide substitutions, 11 of which were predicted to give amino acid changes. The deduced amino acid sequence of VP7 from the OK118 strain was similar to published sequences of a Japanese isolate and three foreign isolates (more than 98.4% identities), whereas the VP7 sequence of the OK450 strain revealed around 96% identities with these isolates and had nine unique amino acid substitutions. The VP7 genes of nine Okayama isolates were then analyzed by dot blot hybridization with the VP7 probes of the OK118 and OK450 strains. Under highly stringent conditions, the OK118 probe produced strong hybridization signals with the genes of five pattern I isolates and one pattern II isolate, while the OK450 probe strongly reacted only with those of three pattern II isolates. These results concluded that relative sequence diversity in the VP7 gene was observed between two different electropherotypes prevalent in a limited area.

TABLE 4. Sources, genome patterns, and the dot blot results of 11 clinical isolates

Code no.	Date of isolation (mo/day/yr)	Age	Sex	Genome pattern	Dot blot result with Probe:	
					OK118	OK450
OK70	1/11/88	1yr	F	I	+	−
OK118	2/ 6/88	6yr	M	I	+	−
OK222	4/ 7/88	5yr	M	I	+	−
OK231	4/25/88	4yr	F	I	+	−
OK239	4/28/88	5yr	F	I	+	−
OK450	2/ 8/89	1yr	F	II	−	+
OK459	2/13/89	1yr	F	I	+	−
OK462	2/13/89	5yr	M	II	−	+
OK595	8/12/89	13yr	M	II	+	−
I54	6/ 7/90	7yr	F	II	−	+
I57	6/13/90	6yr	F	II	−	+

TABLE 5. Nucleotide and deduced amino acid sequence homologies of VP7 genes from six CHRV strains

Strain (country of origin)	% of homology to strain[a]:						Accession no.
	OK450	OK118	88-220	Bristol	Preston	Belém	
OK450 (Japan)		**95.7**	**95.6**	**95.6**	**95.4**	**95.3**	D87544
OK118 (Japan)	96.7		**99.3**	**98.2**	**98.0**	**98.1**	D87543
88-220 (Japan)	96.7	99.4		**98.3**	**98.1**	**98.0**	M61100
Bristol (England)	96.7	98.8	99.4		**99.8**	**97.8**	X77257
Preston (England)	96.7	98.8	99.4	100		**97.8**	X77258
Belém (Brazil)	96.4	98.5	99.1	99.1	99.1		X77256

[a]The percent homologies of nucleotide sequences are given in boldface. Other data represent the percent homologies of deduced amino acid sequences.

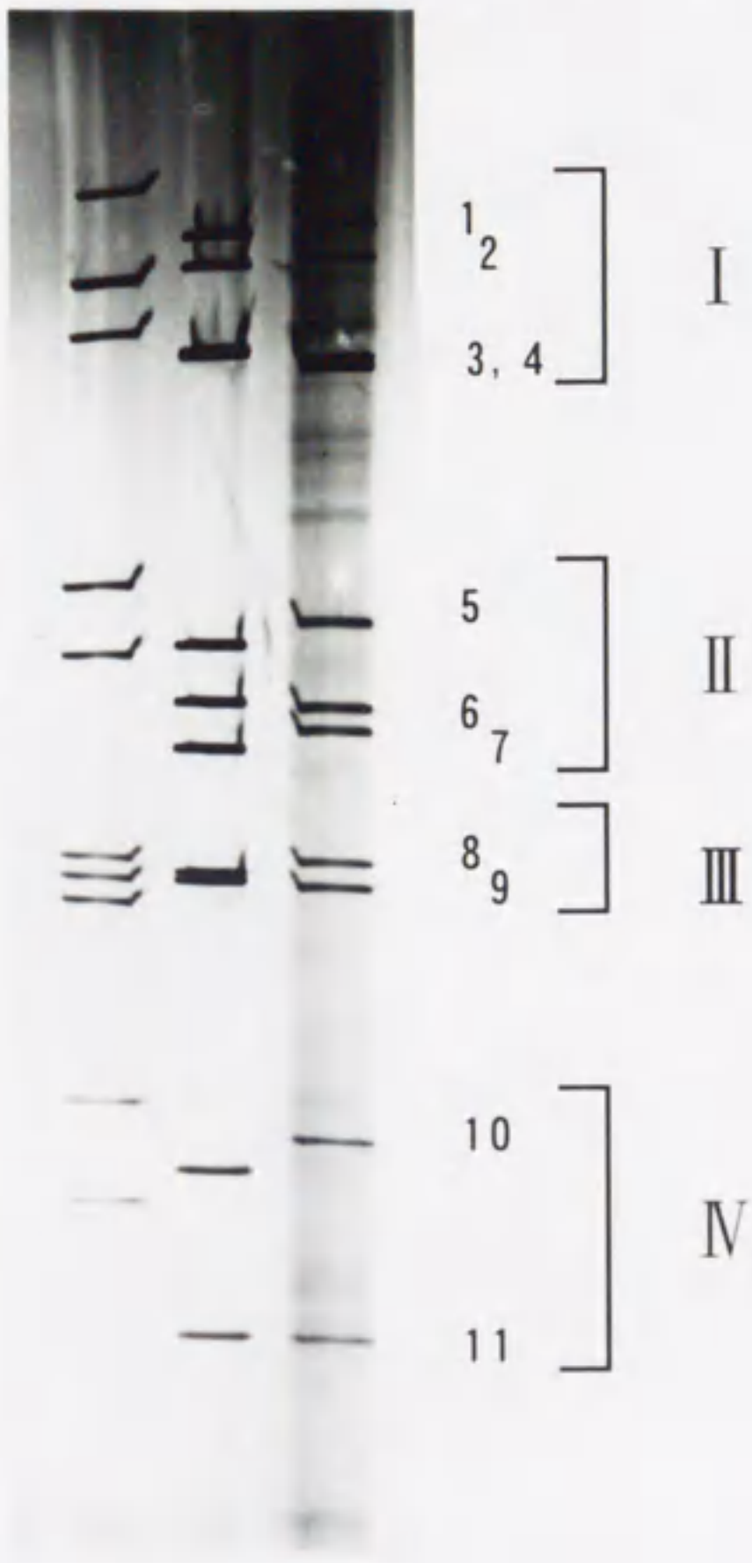

FIG. 4. RNA electropherotypes of human group A and group C rotavirus strains. The viral dsRNAs were dissociated in a 10% polyacrylamide slab gel. The RNA bands were visualized by silver nitrate. Lanes: 1, Wa strain of AHRV; 2 and 3, OK118 and OK450 strains of CHRV, respectively. The approximate positions of RNA segments of OK450 are indicated on the right.

```
OK118    1:GGCATTTAAAAAAGAAGAAGCTGTCTGACAAACTGGTCTTCTTTTTAAATGGTTTGTACAACATTGTACACTGTTTGCGCCATTCTCTTCATTCTTTTCA
OK450      .....................................C.....................................T.......G...........

         101:TTTATATATTATTATTTAGAAAAATGTTCCACCTAATAACTGATACTTTAATAGTGATACTTATTTTATCTAATTGTGTAGAGTGGTCACAAGGTCAGAT
             ........C.....................................................................G...................

         201:GTTTATTGATGATATATATTACAATGGTAACGTTGAGACTATCATAAATTCTACTGATCCTTTTAATGTTGAATCTTTATGTATTTATTTTCCAAATGCA
             ..................C..T.............A.......................C.....................................

         301:ATTGTAGGATCACAAGGACCAGGTAAATCTGATGGACATTTGAATGATGGTAATTATGCACAGACTATCGCCACTTTATTTGAAACAAAAGGATTCCCAA
             G....G.................C.................A.................T........T..T.....G...................

         401:AAGGTTCAATAATACTTAAAACATATACACAGACATCAGACTTTATAAATTCAGTAGAAATGACATGCTCTTATAACATAGTTATCATTCCCGATAGGCC
             .......G...G.....................................................G.............C.................T........

         501:AAATGATTCAGAATCTATTGAACAGATAGCAGAATGGATTTTAAATGTTTGGAGATGTGATGACATGAATTTGGAAATTTATACTTATGAACAAATTGGA
             ............A......G.....................................................................G......

         601:ATAAACAATTTATGGGCTGCATTTGGTAGTGACTGTGATATATCTGTCTGTCCATTAGATACTACAAGTAATGGAATTGGATGTTCACCAGCTAGTACAG
             .....T.....................A......C...........T.................................G...................

         701:AAACTTATGAAGTTGTATCAAATGACACCCAATTGGCATTAATTAATGTTGTGGATAATGTTAGACATAGAATACAGATGAACTCTGCTCAATGTAAATT
             ............................T.....................A.........A..................TA................

         801:AAAAAATTGTATTAAGGGTGAGGCTCGACTGAATACTGCACTAATAAGAATTTCAACATCATCAAGTTTTGATAATTCATTGTCACCATTAAATAACGGC
             .................A...T.......A.................G.....................................................G....

         901:CAAACAACAAGATCGTTTAAAATAAATGCAAAGAAATGGTGGACTATATTTTATACAATAATTGATTATATTAATACAATTGTACAAGCAATGACTCCCA
             ..................................................G........C.................G...............

        1001:GACATCGGGCGATTTATCCAGAAGGGTGGATGTTGAGGTATGCGTAAACAAGATCATGTGGCT
             ..........A.............A.............C.....................
```

39

FIG. 5. Nucleotide sequences of cDNA corresponding to the VP7 genes of the OK118 and OK450 strains. Identical nucleotides are represented by a dot. Initiation and termination codons are underlined.

```
OK118     1:MVCTTLYTVCAILFILFIYILLFRKMFHLITDTLIVILILSNCVEWSQGQMFIDDIYYNGNVETIINSTD
OK450       ..........V..V..................................................
88-220      ..........V..V..................................................
Bristol     ..........V..V..................................................
Preston     ..........V..V..................................................
Belém       ..........V..V..................................................

           71:PFNVESLCIYFPNAIVGSQGPGKSDGHLNDGNYAQTIATLFETKGFPKGSIILKTYTQTSDFINSVEMTC
              ..............V.....................................V..................
              ..............V.....................................V..................
              ..............V.....................................V..................
              ..............V.....................................V..................
              ..............V.....................................V..................

          141:SYNIVIIPDRPNDSESIEQIAEWILNVWRCDDMNLEIYTYEQIGINNLWAAFGSDCDISVCPLDTTSNGI
              ..............T.G.....................................N..................
              .........S.....................................................
              .........S.....................................................
              .........S.....................................................
              .........S.....................................................

          211:GCSPASTETYEVVSNDTQLALINVVDNVRHRIQMNSAQCKLKNCIKGEARLNTALIRISTSSSFDNSLSP
              ..............................K......T............S..................
              ....................................T.....................................
              ....................................T.....................................
              ....................................T.....................................
              ....................................T.........V...........................

          281:LNNGQTTRSFKINAKKWWTIFYTIIDYINTIVQAMTPRHRAIYPEGWMLRYA
              ..S.....................................................
              .....................................................
              .....................................................
              .....................................................
              .....................................................
```

FIG. 6. Alignment of the deduced amino acid sequences of VP7 from the OK118 and OK450 strains with the published sequences from four CHRV strains. Identical amino acids are represented by a dot. Potential N-linked glycosylation sites in the OK118 sequence are boxed.

	PROBE	
	OK118	OK450
OK70 (Ⅰ)	●	
OK118(Ⅰ)	●	
OK222(Ⅰ)	●	
OK231(Ⅰ)	●	
OK239(Ⅰ)	●	
OK450(Ⅱ)		●
OK459(Ⅰ)	●	
OK462(Ⅱ)		●
OK595(Ⅱ)	●	
I 54 (Ⅱ)		●
I 57 (Ⅱ)		●

FIG. 7. Dot blot hybridization of the OK118 and OK450 VP7 probes with amplified VP7 genes from 11 clinical isolates. Equivalent amounts (200 ng) of the genes were spotted on nylon membranes and hybridized with the probe under highly stringent conditions (50% formamide, 5X SSC at 52℃). The genome pattern of each isolate is shown in parentheses.

CHAPTER III

Survey of Human Group C Rotaviruses in Japan during the Winter of 1992 to 1993

INTRODUCTION

Rotaviruses are recognized as the major etiologic agents of diarrheal diseases in young children and animals. The genome of rotaviruses consists of 11 segments of dsRNA enclosed in a double-shelled particle. Rotaviruses are classified into seven groups (groups A to G) on the basis of their dsRNA electropherotypes and a common group antigen on the inner-capsid protein (protein VP6) (36).

Group C rotaviruses were first recognized in swine (2, 38), and then were confirmed as human pathogens by Bridger et al. (4). During the last decade, CHRVs have been associated with several outbreaks of acute diarrhea in Asia (25, 33), Europe (3, 6) and South America (13). More recently, CHRVs have been detected in patients with sporadic cases of diarrhea in the United States (20). These observations indicate that CHRV is widely distributed and is likely to be an emerging pathogen.

CHRV infection in Japan was first recognized by Oseto et al. (30) in 1985. Ushijima et al. (47) also detected CHRVs from fecal specimens collected in the Tokyo area in 1987. Since then, some researchers have recognized CHRV infections in individual locations (12, 27, 29). However, none of these investigators have carried out an epidemiological study that covers several locations in Japan.

Until recently, the detection of CHRVs in fecal specimens was usually performed by immune electron microscopy with CHRV-specific antisera or polyacrylamide gel electrophoresis (PAGE) of viral dsRNA, because CHRVs were noncultivatable. The complicatedness in detection has hindered the epidemiological analysis of CHRV infection. In Chapter I, I have developed a RPHA test using CHRV-specific MAbs. This test is very easy to perform and should be a useful tool for the screening of CHRVs on a large scale.

In this chapter, for the epidemiological study of CHRV in Japan, fecal specimens from patients with diarrhea were collected in 10 prefectures and were examined for the presence of CHRVs by the RPHA test. As a result, CHRVs were detected in seven prefectures. To define the genetic relationship among the CHRV clinical isolates, I analyzed the genome electropherotypes and the VP7 gene homology of the isolates.

MATERIALS AND METHODS

Fecal specimens

Between November 1992 and April 1993, 1,114 fecal specimens from patients with acute diarrhea were collected at pediatric clinics or outpatient sections of general hospitals in the following 10 prefectures: Chiba (n = 97), Niigata (n = 55), Toyama (n = 119), Gifu (n = 74), Fukui (n = 21), Tottori (n = 123), Okayama (n = 176), Kagawa (n = 163), Shimane (n = 187) and Saga (n = 99). Figure 8 shows the geographic areas in which the specimens were collected.

All fecal specimens were screened for AHRV with an ELISA kit (ROTACLONE; Cambridge Biotech). The specimens that were negative for AHRV were further examined for the presence of CHRV by the RPHA test.

RPHA test

The RPHA test was performed as described in Chapter I. Briefly, 10% suspensions of the fecal specimens in phosphate buffered saline (pH 7.2) were centrifuged at 2,000 X g for 10 min and the supernatants were subjected to the test. Serial twofold dilutions of the samples were made in duplicate. In one dilution series, a 0.7% suspension of sheep red blood cells (SRBCs) coated with CHRV-specific MAbs was added to each well. In the other series, SRBCs coated with normal mouse Immunoglobulin G (control SRBCs) were added. Hemagglutination titers were observed after 1 h. When the RPHA test titer with MAb-coated SRBCs was four or more times greater than that obtained with the control SRBCs, the sample was judged to be CHRV positive.

RNA extraction

Fecal suspensions containing CHRV were extracted with an equal volume of trichlorotrifluoroethane, and the supernatants were adjusted to contain 10 mM EDTA,

0.6% SDS and 300 μg of proteinase K per ml. The suspensions were incubated for 1.5 h at 40℃ and then extracted with phenol-chloroform. Viral dsRNA was further purified with an RNAID kit (BIO 101, Inc.) according to the manufacturer's instructions. The purified RNAs were stored at -30℃ until use.

Dot blot hybridization

Hybridization tests were carried out as described in Chapter II. In brief, the VP7 genes of CHRV isolates were first amplified by the RT-PCR method and were then purified with a Suprec-02 column (Takara Shuzo Co., Ltd.). Equivalent amounts (200 ng) of the genes amplified by RT-PCR were blotted onto nylon membranes, and were separately hybridized with K9304, OK118 and OK450 VP7 genes that had been labeled with digoxigenin-11-dUTP (Boehringer). Hybridization was performed under highly stringent conditions (50% formamide and 5 X SSC at 52℃). After hybridization, the membranes were washed twice in 0.1×SSC containing 0.1% SDS at 68℃ for 15 min. Colorimetric detection of the hybridized probe was carried out according to the manufacturer's instructions.

Sequencing of the VP7 gene

The VP7 gene of a clinical isolate (strain K9304) was amplified by the RT-PCR method and was then cloned into plasmid pUC18. Five individual recombinants were isolated, and both strands of the cloned DNA were sequenced by the dideoxynucleotide chain-termination method (Applied Biosystems). Nucleotide sequence data were then analyzed by the GENETYX-MAC, version 6.0, program. The program MAlign, version 1.0, was also used for sequence alignments.

Nucleotide sequence accession number

The nucleotide sequence data for the VP7 gene of the K9304 strain has been submitted to the DDBJ DNA database and has been assigned accession number AB004250.

RESULTS

Detection of CHRVs from fecal specimens by the RPHA test

The results of the detection of rotaviruses are summarized in Table 6. AHRVs were detected in 330 (29.6%) of 1,114 specimens by the ELISA kit. Group A rotavirus-negative samples (n = 784) were further examined for CHRVs by the RPHA test. As a result, a total of 53 specimens (6.8%) were positive for CHRV. These specimens were obtained in 7 of the 10 prefectures. The geographic distribution of the prefectures in which CHRVs have been detected is shown in Fig. 8. CHRVs were mainly distributed in the western area of Japan. The positive rates of CHRV ranged from 2.7% (Chiba) to 13.3% (Shimane) and were considerably lower than those of AHRV. No significant difference was observed between the positive rates of males and females (data not shown).

The epidemiological features of CHRV infection were then compared with those of AHRV infection. CHRVs were mainly detected in March and April (Table 6), whereas most AHRV infections occurred between February and March (data not shown). The age-specific attack rates for human group C and group A rotaviruses are compared in Table 7. Although CHRVs principally prevailed in children ages 3 to 8 years, the target age groups of AHRV were less than 3 years. The mean age of the patients infected with CHRV (4.36 years) was significantly ($P < 0.01$) higher than that of patients infected with AHRV (2.19 years).

Genome electropherotypes of CHRV isolates

Only eight specimens which were obtained from five prefectures had enough volume from which viral dsRNA could be extracted for genome electropherotype analyses. The RNA segments were dissociated by PAGE with a 10% gel and were then visualized with silver nitrate. Figure 9 (lanes 1 to 8) shows the genome electropherotypes of the clinical

isolates. Every isolate exhibited the typical 4-3-2-2 profile of group C rotavirus. Although the isolate were detected in five individual prefectures, the electropherotypes of all isolates were similar to each other.

The author demonstrated that the genome electropherotypes of CHRVs isolated in Okayama from 1988 to 1990 were classified into patterns I and II in Chapter 2. The electropherotype of the K9304 strain, which was selected as a representative of the isolates retrieved in 1993, was then compared with those of the OK118 (pattern I) and the OK450 (pattern II) strains in the same gel (Fig. 9, lanes 9 to 11). The electropherotype of the K9304 strain was different from those of both the OK118 and OK450 strains, and so this electropherotype was tentatively designated pattern III. The electrophoretic mobilities of the 2nd, 3rd, 7th, 10th and 11th segments of the K9304 strain were similar to those of the corresponding segments of the OK118 strain, while the 1st, 4th, 5th and 6th segments migrated to the same positions as those of the OK450 strain, suggesting that pattern III is a combined electropherotype of patterns I and II.

Analysis of the VP7 genes from CHRV isolates

The VP7 gene homology of the clinical isolates was further analyzed by dot blot hybridization with VP7 gene probes from the K9304, OK118 and OK450 strains. Epidemiological data and the hybridization results for the 19 isolates used in the study are presented in Table 8. The VP7 genes of all isolates strongly reacted with the K9304 probe as well as the OK118 probe, while weak hybridization signals were observed with the OK450 probe. These results indicate that the VP7 genes of the isolates retrieved in 1993 have high levels of homology not only with each other but also with the VP7 gene of the OK118 strain, which was isolated in 1988.

To confirm these findings, the VP7 gene of the K9304 strain was cloned and sequenced. The VP7 gene of the K9304 strain was 1,063 nucleotides in length and contained single

open reading frame encoding 332 amino acids. The nucleotide and deduced amino acid sequences of the VP7 gene from the K9304 strain were further compared with those of the genes from the OK118 and OK450 strains. As indicated in Table 9, a surprising level of sequence conservation was observed between the K9304 and OK118 strains (more than 99.1%), whereas the overall nucleotide and amino acid identities between the K9304 and OK450 strains were relatively low (95.6 and 96.7%, respectively).

DISCUSSION

This is the first study of a survey of CHRVs in various locations in Japan. A total of 53 isolates were detected in 7 of 10 prefectures that were surveyed, indicating that CHRVs are widely distributed over Japan. The rates of CHRV positivity ranged from 2.7% to 13.3%, and the CHRV isolates were mainly detected in March and April. Moreover, CHRVs principally prevailed in children ages 3 to 8 years. These epidemiological features are distinct from those of the AHRV infection. Oseto (29) previously carried out an epidemiological study of CHRV over a 3-year period in Matsuyama City, Japan. The epidemiological features observed in this study were consistent with those reported by Oseto (29).

In this study, the group A rotavirus-negative specimens were only screened for the presence of CHRV. Jiang et al. (20) have recently reported an mixed infection with human group A and group C rotaviruses in the United States. Therefore, group A rotavirus-positive specimens (n = 52) that were collected in Okayama and Shimane were examined by the RPHA test (data not shown), but none of the specimens was positive for CHRV, indicating that the mixed infection might be rather rare. In fact, Jiang et al. (20) recognized only one mixed infection among 1,676 samples. However, screening of rotavirus infections must hereafter include screening for mixed infection.

The RPHA test could successfully detect CHRVs even in fecal specimens that were insufficient for immune electron microscopy or genome electropherotype analyses. To inspect the specificity of the RPHA test, the RPHA test-positive specimens were examined by the ELISA test with CHRV-specific MAbs (12), and all were determined to be positive. Recently, the PCR method has been applied to the detection of group C rotaviruses by Gouvea et al. (15). Although the sensitivity of the RPHA test is not comparable to that of

the PCR method, the former test is faster and simpler and is more suitable for routine diagnosis in clinical settings.

In group A rotavirus, genome electropherotyping of clinical isolates is a useful tool for obtaining epidemiological information about the origin of the isolates and diversity among these isolates, because each isolate reveals a unique genome profile (1, 43). The genome electropherotypes of the isolates retrieved in 1993 were surprisingly similar to each other, regardless of the prefectures from which the isolates were obtained. Moreover, the dot blot hybridization analysis showed that the VP7 genes of the isolates were highly homologous. These results strongly suggest that a large-scale outbreak of CHRV occurred during the winter of 1992 to 1993 in Japan. However, further comparative analysis of other genome segments will be required to confirm this hypothesis.

The electropherotype of the K9304 strain, which represented the isolates retrieved in 1993, was compared with those of the pattern I and II strains in the same gel. Although the K9304 strain revealed a distinct genome profile tentatively designated pattern III, this electropherotype seemed to be a combination of patterns I and II. The sequence analysis of the VP7 gene from the K9304 strain also showed that the gene was similar to that of the pattern I strain. These results indicate that the K9304 strain may be a reassortant virus between the pattern I and pattern II strains, because it has been reported that natural reassortants occurred between group A rotavirus strains belonging to different genogroups (26, 48). Quite recently, CHRVs have been successfully propagated in a continuous cell line (CaCo-2) (28, 42). To clarify the genetic and antigenic relationship among three strains with distinct electropherotypes, it will be needed to adapt these strains to CaCo-2 cells.

SUMMARY

Fecal specimens from patients with acute diarrhea were collected from 10 prefectures in Japan over a 6-month period (November 1992 to April 1993), and the specimens that were negative for group A rotavirus were screened for the presence of CHRVs by the RPHA test. Of 784 specimens examined, 53 specimens (6.8%) that were collected in 7 of 10 prefectures were positive for CHRV, indicating that CHRVs are widely distributed across Japan. Most of the isolates were detected in March and April, and CHRVs mainly prevailed in children ages 3 to 8 years. The genome electropherotypes of eight isolates detected in five individual prefectures were surprisingly similar to each other and were different from those of CHRVs isolated to date. The outer capsid glycoprotein (VP7) gene homology of the isolates retrieved in 1993 was subsequently analyzed by the dot blot hybridization method. As a result, the VP7 genes of the isolates revealed very high levels of homology not only with each other but also with the VP7 gene of the OK118 strain isolated in 1988. These results suggest that a large-scale outbreak of CHRV occurred during the winter of 1992 to 1993 in Japan.

TABLE 6. Detection of rotaviruses in 10 prefectures

Prefecture	Detection result[a] of:		No. of CHRV isolates detected in:					
			1992		1993			
	AHRVs[b]	CHRVs[c]	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.
Chiba	24/97 (24.7)	2/73 (2.7)					1	1
Niigata	7/55 (12.7)	0/48						
Toyama	49/119(41.2)	0/70						
Gifu	20/74 (27)	0/54						
Fukui	6/21 (28.6)	2/15 (13.3)						2
Tottori	46/123(37.4)	6/77 (7.8)						6
Okayama	22/176(12.5)	11/154(7.1)		2		2	3	4
Kagawa	83/163(50.9)	8/80 (10)		2	1	1	3	1
Shimane	30/187(16)	19/157(12.1)	1		4	6	6	2
Saga	43/99 (43.4)	5/56 (8.9)			2	2	1	
Total	330/1,114(29.6)	53/784(6.8)	1	4	7	11	14	16

[a]Values show no. of samples that were positive/total no. of samples tested. Values in parentheses show percent of tests that were positive.

[b]AHRVs were detected with a commercially available ELISA kit.

[c]CHRVs were detected by the RPHA test.

TABLE 7. Comparison of age-specific attack rates for CHRV and AHRV

Age group (years)	Attack rate (%)	
	CHRV	AHRV
<1	4.2	38.7
1 - 2	8.9	42.3
3 - 4	12.8	24.2
5 - 6	6.2	14.5
7 - 8	10.0	10.7
9 -10	4.2	4.0
>10	2.6	10.0

TABLE 8. Epidemiological data, genome patterns, and dot blot hybridization results of 19 CHRV isolates

Code no.	Date of isolation (mo/day/yr)	Age	Sex	Prefecture	Genome pattern	Dot blot result with probe: K9304	OK118	OK450
OK118	2/ 6/88	6yr	M	Okayama	I	+	+	−
OK450	2/ 8/89	1yr	F	Okayama	II	−	−	+
93K332	3/23/93	24yr	F	Chiba	III	+	+	−
93K360	4/27/93	9yr	M	Chiba	III	+	+	−
F522	4/30/93	1yr	M	Fukui	III	+	+	−
F618	4/24/93	4yr	F	Fukui	ND[a]	+	+	−
34745	4/23/93	15yr	M	Tottori	ND	+	+	−
34803	4/12/93	1yr	F	Tottori	III	+	+	−
T360	3/ 6/93	3yr	M	Okayama	III	+	+	−
K12	3/15/93	6yr	M	Okayama	ND	+	+	−
K9301	4/19/93	8yr	M	Okayama	ND	+	+	−
K9304	4/22/93	4yr	M	Okayama	III	+	+	−
K9306	4/26/93	4yr	F	Okayama	ND	+	+	−
KA63	3/ 3/93	7yr	M	Kagawa	ND	+	+	−
KA73	3/30/93	5yr	M	Kagawa	ND	+	+	−
S373	1/25/93	1yr	F	Shimane	ND	+	+	−
S891	2/ 2/93	1yr	M	Shimane	ND	+	+	−
93-42	2/ 2/93	1yr	M	Saga	III	+	+	−
93-48	2/23/93	1yr	M	Saga	III	+	+	−

[a]ND, genome pattern could not be determined.

TABLE 9. Nucleotide and deduced amino acid sequence homologies of VP7 genes from three CHRV strains

Strain	% of homology to strain[a]: K9304	OK118	OK450	Accession no.
K9304		**99.2**	**95.6**	AB004250
OK118	99.4		**95.7**	D87543
OK450	96.7	96.7		D87544

[a]The percent homologies of nucleotide sequences are given in boldface. Other data represent the percent homologies of deduced amino acid sequences.

FIG. 8. Map of Japan showing the prefectures from which the fecal specimens were collected. Daker shading indicates the prefectures in which CHRVs have been detected. A, Chiba; B, Niigata; C, Toyama; D, Gifu; E, Fukui; F, Tottori; G, Okayama; H, Kagawa; I, Shimane; J, Saga.

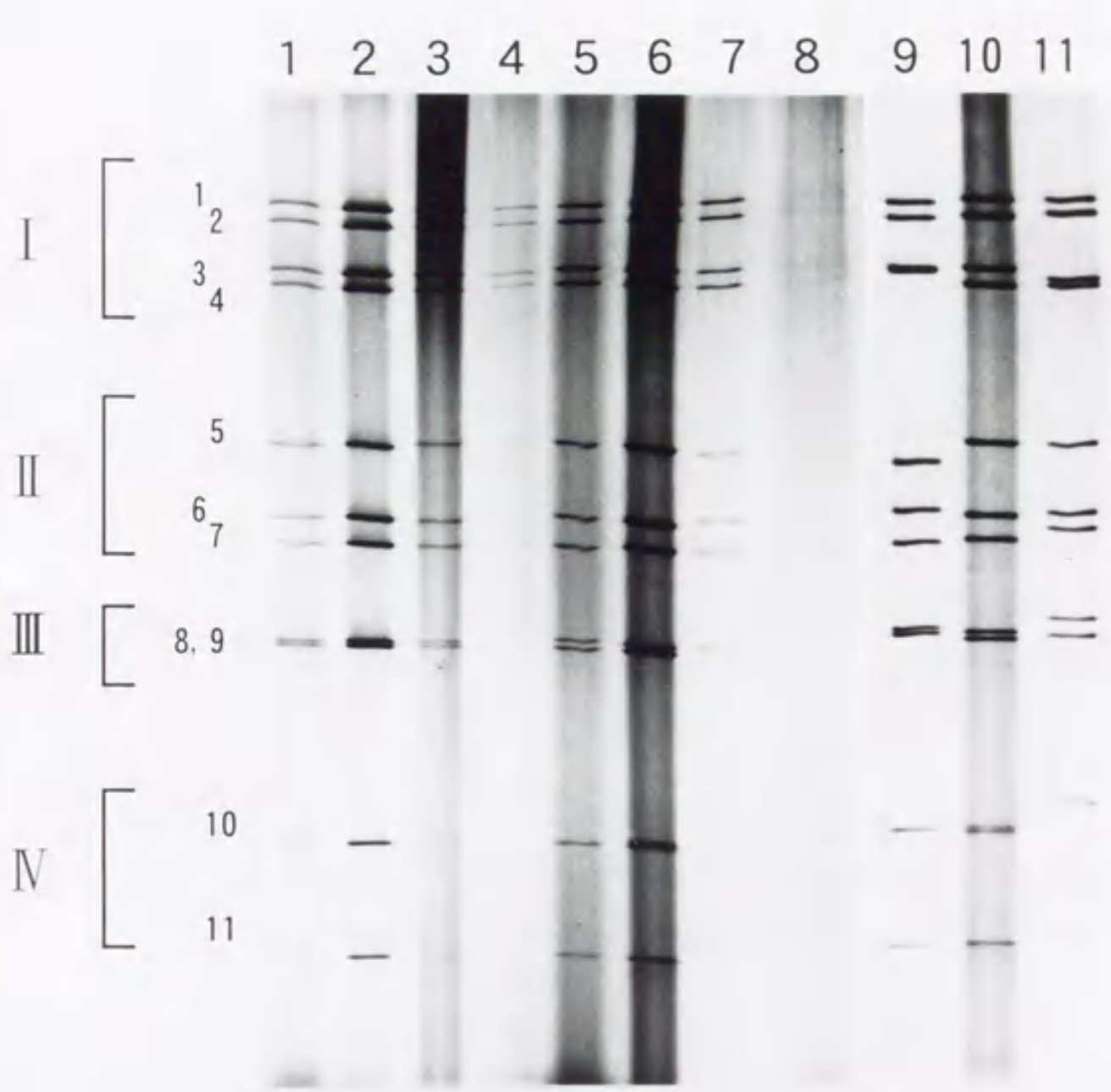

FIG. 9. Comparison of genome electropherotypes of CHRV clinical isolates. Lanes: 1, 93K332 strain (Chiba isolate); 2, 93K360 strain (Chiba isolate); 3, F522 strain (Fukui isolate); 4, 34803 strain (Tottori isolate); 5, T360 strain (Okayama isolate); 6, K9304 strain (Okayama isolate); 7, 93-48 strain (Saga isolate); 8, 93-42 strain (Saga isolate); 9, OK118 strain which exhibits the pattern I electropherotype; 10, K9304 strain which exhibits the pattern III electropherotype; 11, OK450 strain which exhibits the pattern II electropherotype.

CONCLUSION

Human group C rotavirus (CHRV) is known to be globally distributed and is thought to be one of emerging pathogens in humans. Detection of CHRV has usually been done by immune electron microscopy (IEM) or by polyacrylamide gel electrophoresis of viral RNA (RNA-PAGE), because this virus is noncultivatable. The complicatedness in detection has hindered the epidemiological analysis of CHRV infection. In order to simplify the detection of CHRV, the author developed rapid and specific diagnostic methods. Furthermore, the genomes of CHRV clinical isolates have been analyzed in molecular level, because knowledge of the genetic variation among CHRVs is limited. Some observations which the author has newly obtained are as follows.

Reverse passive hemagglutination (RPHA) and latex agglutination (Lx-Ag) tests using anti-CHRV monoclonal antibodies (MAbs) were developed for the rapid and specific detection of CHRV. By these methods, CHRVs have been successfully detected from fecal specimens. The minimum numbers of CHRV detectable by the RPHA and Lx-Ag tests were 1.5×10^{7} and 3.0×10^{8} particles per ml, respectively. The present methods have several obvious advantages over IEM and PAGE. The first advantage is detection time. It takes only 2 min to detect CHRV by the Lx-Ag test and 3 h by the RPHA tests. The second advantage is the simplicity of the test systems. Once SRBCs and Lx coated with MAb are ready, the reactions involve a single step. No special equipment is required for the Lx-Ag test and the only equipment required for the RPHA tests is a microtitration set. The present methods would be therefore valuable for the gathering of epidemiological data to establish the importance of CHRV in human diarrheal disease and also be suitable for small clinical laboratories which are not equipped well.

The author showed that two different electropherotype isolates, which were classified

into patterns I and II, were detected in Okayama Prefecture during a 1988 to 1990 epidemic and that dominant patterns changed from year to year. Two clinical isolates (OK118 and OK450) were selected as prototypes of patterns I and II, respectively, and a comparative nucleotide and amino acid sequence analysis of the VP7 gene of these isolates was performed. Although the sequences were identical in size (1,063 bp) and both contained single open reading frames with the same size, there were 46 nucleotide substitutions between them. The comparison of the predicted VP7 sequence also showed that amino acid substitutions occurred at 11 positions. Dot blot hybridization analysis was performed to evaluate the existence of the VP7 gene divergence represented by the OK118 and OK450 strains in CHRV clinical isolates. As a result, it was revealed that almost exclusive cross-hybridization of the OK118 and OK450 cDNA probes to the clinical isolates with patterns I and II, respectively. Thus, relative sequence diversity in the VP7 gene exists between the two electropherotypes was suggested.

In order to carry out an epidemiological study of CHRV infection in Japan, 1,114 fecal specimens from patients with diarrhea were collected in 10 prefectures over a 6-month period. The specimens that were negative for group A rotavirus were examined for the presence of CHRVs by the RPHA test. As a result, a total of 53 isolates were detected in 7 of 10 prefectures that were surveyed, indicating that CHRVs are widely distributed across Japan. The CHRV-positive rates ranged from 2.7% to 13.3%, and most of the isolates was detected in March and April. CHRVs mainly prevailed in children ages 3 to 8 years. These epidemiological features are distinct from those of human group A rotavirus infection. To define the genetic relationship among the CHRV clinical isolates, the genome electropherotypes and the VP7 gene homology of the isolates was analyzed by PAGE and the dot blot hybridization method, respectively. The genome electropherotypes and the VP7 genes of the isolates were surprisingly similar to each other,

regardless of the prefectures from which the isolates were obtained, suggesting that a large-scale outbreak of CHRV occurred during the winter of 1992 to 1993 in Japan. Moreover, the electropherotype of the K9304 strain, which represented the isolates retrieved in 1993, was compared with those of the pattern I and II strains. Although the K9304 strain revealed a distinct genome profile tentatively designated pattern III, this electropherotype seemed to be a combination of patterns I and II. The sequence analysis of the VP7 gene from the K9304 strain also showed that the gene was similar to that of the pattern I strain. These results suggest that the pattern III virus is a reassortant virus between the pattern I and II viruses.

In this treatise, the author developed the RPHA and Lx-Ag tests for the rapid and specific detection of CHRVs. By using the RPHA test for the epidemiological study that covers several locations in Japan, the author defined that CHRVs are widely distributed across Japan and that epidemiological features of CHRV infection are distinct from those of human group A rotavirus infection. Moreover, the author demonstrated that genetic divergence existed among the CHRV clinical isolates. These observations should be a valuable information for the epidemiology of CHRV infection.

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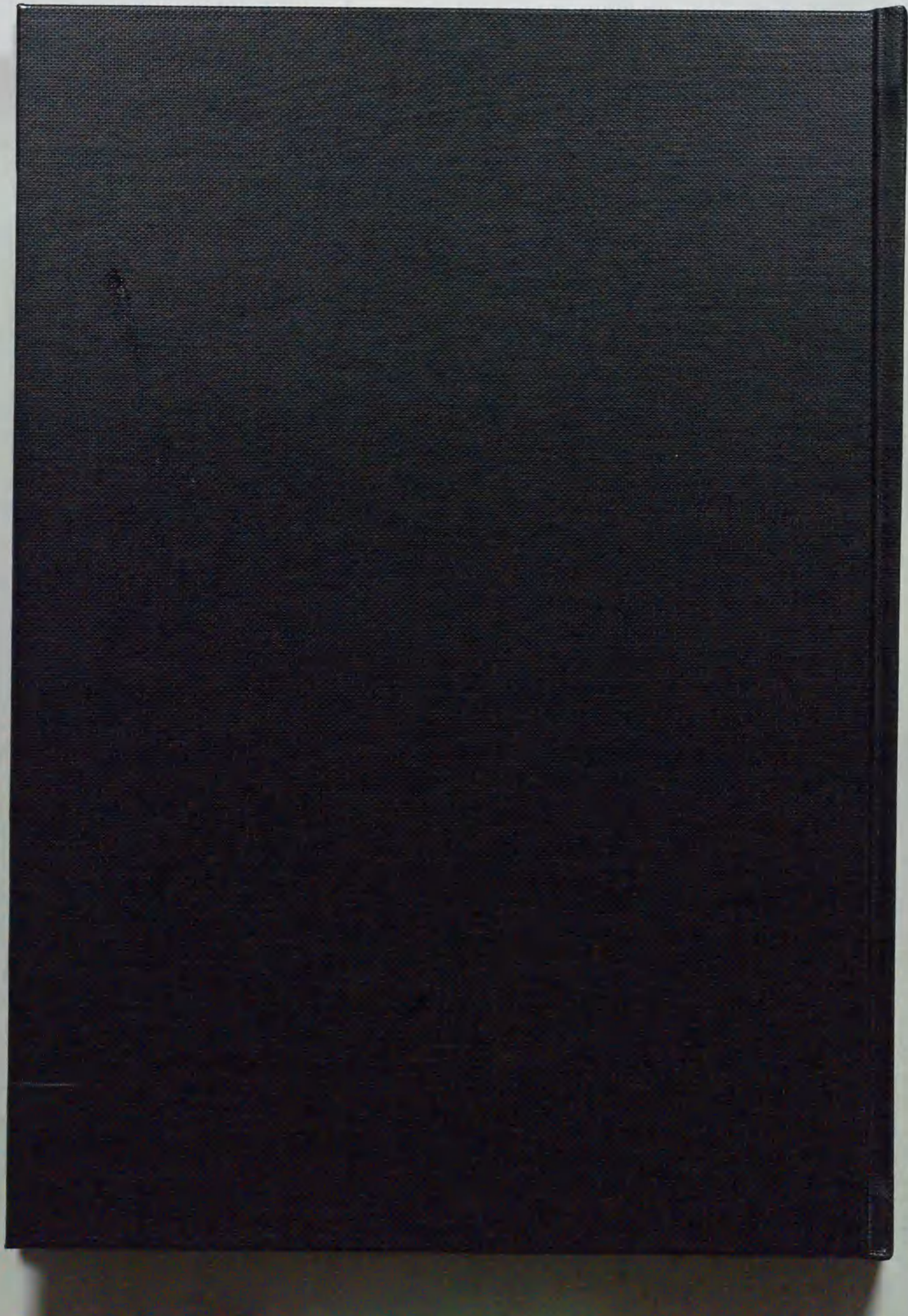

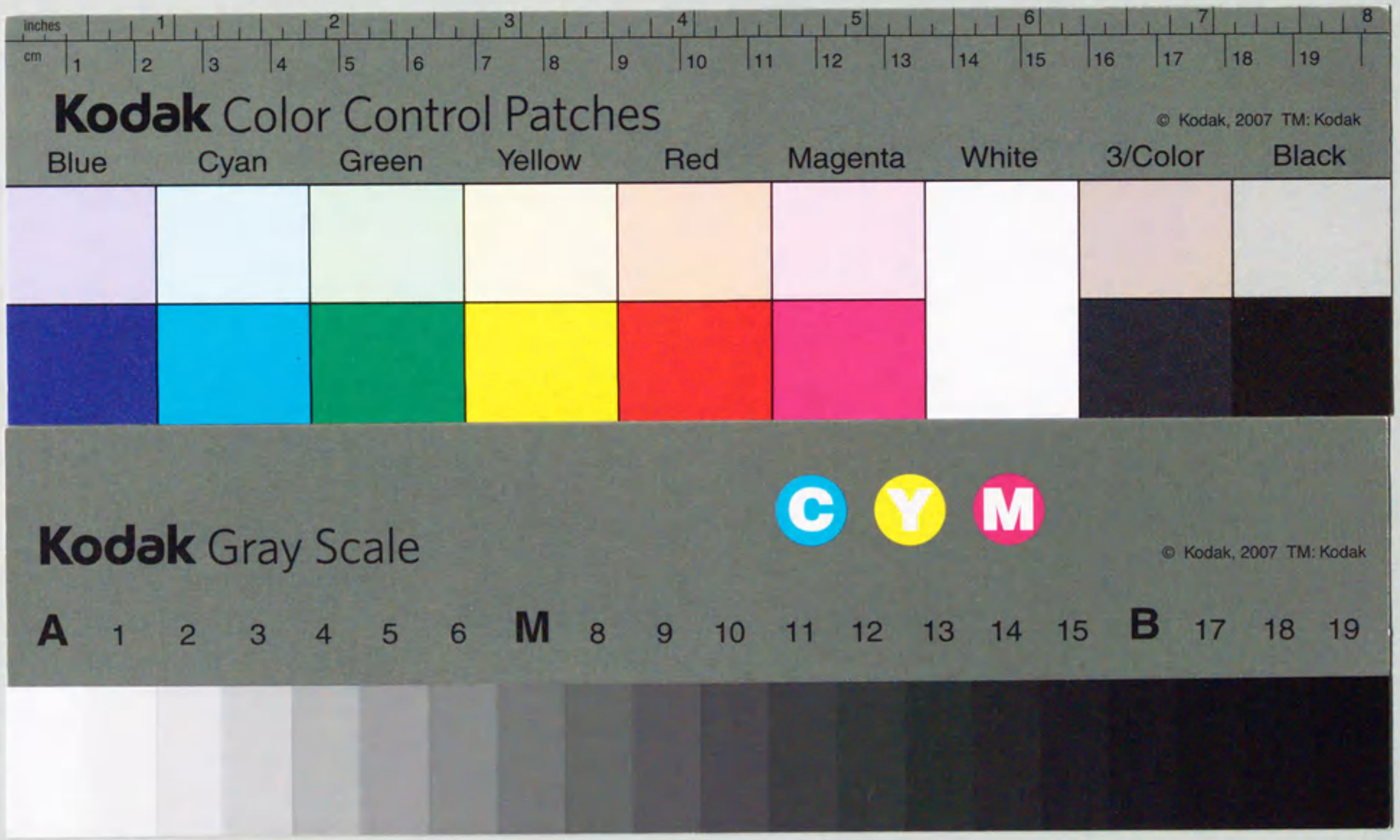
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