緒　言

黄色ブドウ球菌 (*Staphylococcus aureus*) を原因とする食中毒は，おにぎりなどの穀類およびその加工品，弁当，惣菜など複合調理食品，菓子類などの種々の食品によることが報告されている[20]．食品従事者の衛生管理や施設設備の改善（機械化，自動化）が進み，食中毒発生件数は減少傾向にある[10]．しかし，依然としておにぎりや弁当により多数の患者が発生する事件もしばしば見られる．仕出し屋や飲食店で製造される弁当類は手作業で調理される場合がほとんどであり，手指や器具・機器からの *S. aureus* 汚染を確実に遮断することが求められる．よって，調理施設における *S. aureus* の汚染実態，伝播実態を把握しておくことは食中毒予防対策上極めて重要であると考えられる．作業者の手指，まな板，水道蛇口カラン，食品などにより病原菌が伝播することもモデル実験で示されている[3,15,16,31]が，実際の調理作業で *S. aureus* が伝播する実態を把握した研究は少ない[12]．

これまで *S. aureus* の汚染経路などの解明にはコアグラーゼ型，エンテロトキシン (SE) 型などが利用されてきたが，最近細菌感染症の疫学調査や汚染原因菌調査に分子生物学的手法，すなわち DNA の多様性により分類する遺伝子多型解析法が多用されており，特に院内感染 *S. aureus* として問題になるメチシリン耐性菌 (MRSA) の疫学的研究については顕著である[1,2,11,22,23]．

S. aureus の遺伝子多型解析法には，Randomly Amplified Polymorphic DNA analysis (RAPD) 法（または AP-PCR 法）[1,5,23]，パルスフィールドゲル電気泳動 (Pulsed-Field Gel Electrophoresis, 以下 PFGE) 法[9,22,23,26]，Multilocus Sequence Typing (MLST) 法[6,29]，リボタイピング法[9,21,22]，Insertion Sequence (IS) タイピング法[4,30]，Amplified Ribosomal DNA Restriction Analysis (ARDRA) 法[7]などが用いられている．

PFGE 法による型別は多数報告され，その妥当性，安定性，再現性が高く評価されており[14,23]，本法は泳動原理別に CHEF (Contour-clamped homogeneous electric field gel electrophoresis) 法，バイアス正弦電場ゲル電気泳動（Biased sinusoidal field gel electrophoresis, 以下 BSFGE）法[13]など 7 つに分類されている．

本研究の目的は，環境由来の *S. aureus* が実際の調理作業中に伝播する実態を把握し，その動態を明らかにすること，そして伝播実態調査における遺伝子多型解析法の有用性を評価，検証することである．

材料および方法

1. *S. aureus* の分離

調理実習生 8 グループ 26 名が使用した実習室の各部所および実習生について *S. aureus* の分離を行った．調理前および調理中の実習生の手指からの試料採取はグローブジュース法[28]により行った．食品原材料 28 種，調理済み食品 26 検体は常法[17]により一定量を採取，水道カラン，フライパンなどの実習設備および器具はふき取り法により，また，包丁と菜箸については滅菌処理したものを実習生に使用させ，これを滅菌バッグに入れ，グローブジュース法と同様にサンプリング液を加えて器具表面を十分に洗い，これを試料液として用いた．

2. 分離培養

得られた各試料液は 10 倍段階希釈し，卵黄加マンニット食塩培地（日水製薬）に 0.1 m*l* 塗抹後，35℃, 48 時間培養を行った．黄色で光沢の白濁環を示す集落を釣菌，純培養を行った．

3. *S. aureus* の同定

分離菌株をグラム染色（B&M 山中変法; Merck Japan），コアグラーゼ試験（ウサギプラズマ栄研; 栄研化学）を行い，さらに本菌同定キット (APISTAPH; Biomerieux) を用いて *S. aureus* の同定を行った．分離した菌株は遺伝子多型解析を行うまで −80℃ 冷凍庫で保存した．

4. RAPD 法

(1) 使用プライマー

10 塩基のオリゴヌクレオチドプライマー A-03[2]: 5′-AGTCAGCCAC-3′ と F-04[2]: 5′-GGTGATCAGG-3′ (RAPD 10 mer Kits; QIAGEN) を使用した．

(2) ゲノム DNA の調製

分離した *S. aureus* を TSB 寒天培地（日水製薬）に塗抹し，一夜培養後形成した集落から一白金耳量を採取し，「Gen とる君™（酵母用）」(TaKaRa) を用いてゲノム DNA を分離した．分離ゲノム DNA は TE バッファー（10 mM Tris-HCl, 1 mM EDTA; シグマアルドリッチジャパン）に溶解し −20℃ で保存した．

(3) PCR 反応

AccuPower® PCR PreMix (*Taq*DNA polymerase: 1 U, dNTP: 250 μM, Tris-HCl (pH 9.0): 10 mM, KCl: 40 mM, $MgCl_2$: 1.5 mM; Bioneer) にゲノム DNA 10 μl, 40 pmol のプライマー A-03 またはプライマー F-04 1 μl, 5 mM $MgCl_2$ 1.2 μl と蒸留水を加え総量 20 μl とした．PCR 反応液中の Mg イオン濃度については 1.5, 2.0, 3.0, 4.0 mM の各濃度で予備試験行い，3.0 mM および 4.0 mM で良好な泳動像が得られることを確認したのち 3.0 mM を用いた．PCR 反応は EZcycler（EZC-96, 旭テクノグラス）を用い，藤本らの方法[5]に準じて行った．PCR 増幅物の確認は，2.0% アガロースゲル (AgaroseH14「TAKARA」; TaKaRa) で電気泳動（分子量マーカー: PCR Markers (50–2,000 bp); Novagen）し，エチジウムブロマイド (0.5μg/m*l*) で 20 分間染色し，精製水で 30 分間脱色後撮影により行った．

5. BSFGE 法

分離菌株を SCD 寒天培地（日水製薬）に塗抹後一夜培養した．形成集落の一白金耳量を TSB 培地（栄研器材）に接種し，5 時間培養後，遠心分離（8,000 rpm, 10 分間）を行い，沈査に 0.5 M EDTA を加え，次いでリゾスタフィン (SIGMA) とアガロース (BMA) を加えて，鋳型でブロックに固めた．このブロックに溶菌液（リゾスタフィン＋0.5 M EDTA（和光純薬工業））1 m*l* を加え，37℃ で一夜保存して溶菌させた．次に，タンパク質分解液（プロテイナーゼ K (Sigma)＋1% *N*-ラウロイルサルコシンナトリウム (Sigma)＋0.5 M EDTA）に置換し，50℃ で 8 時間反応させた後，TE バッファーに置換して，プロテイナーゼ K を不活化させた．

ブロックを氷上で制限酵素用バッファー (*Sma*I buffer; TOYOBO) で 30 分間振とうした後，*Sma*I 処理（30℃, 16 時間）を行った．

次に，このブロックを 1% 電気泳動用アガロースゲル (SeaKemGTG; BMA) にセットし TBE バッファー（4.45 mM Tris-borate, 1 mM EDTA; シグマアルドリッチジャパン）中で泳動 (GENOFIELD; ATTO) を行った．泳動は DC＝42 V, AC＝250 V, start＝0.01 Hz, end＝0.2 Hz の条件で 20℃, 24 時間行った．なお，DNA 分子量マーカーとして λ-DNA (Lambda DNA Ladders; CAMBREX) と λ-*Hin*dIII digest (TaKaRa) を用いた．

泳動後ゲルはエチジウムブロマイド (0.5 μg/m*l*) で 30 分間染色後，精製水で 1.5 時間脱色し，撮影により観察した．

6. PFGE 法

PFGE（CHEF 法による）法は，*S. aureus* からの DNA 抽出および制限酵素処理については BSFGE 法と同様に行い，得られた寒天ブロックを 1% 電気泳動用アガロースゲル (Agarose; Invitrogen) にセットし TBE バッファー中で泳動 (CHEF-DR®III; Bio-Rad) を行った．DNA 分子量マーカーとして，緑膿菌の *Sma*I 切断 DNA 断片を用いた．各断片のサイズは λ ラダーの泳動度を比較し算出した．泳動はパルスタイム 1～50 秒，電圧 6.0 V/cm, 内角 120° の条件で，14℃, 22 時間行った．泳動後ゲルはエチジウムブロマイド (2 μg/m*l*) で 30 分間染色後，精製水で 2 時間脱色し，撮影により観察した．

結　　果

1. *S. aureus* の分離と同定

実習および実習中の実習生手指各々26 名からのふき取り材料 26 検体，食品原材料 28 検体，調理済み食品 26 検体，実習用調理器具および設備のふき取り材料 56 検体，計 162 検体について *S. aureus* を検査した結果，15 検体が陽性を示し，これら検体から *S. aureus* 64 株を分離した (Table 1)．これらのうち水道カランとフライパンからは増菌培養により陽性であり，9 株を分離した．分離した 64 菌株はグラム染色陽性，コアグラーゼ反応陽性で，同定キットによりすべて *S. aureus* であった．

2. RAPD パターンと多型解析

プライマー A-03 およびプライマー F-04 を用いて，それぞれ RAPD を行った場合のパターンと分離した *S. aureus* を Table 2 および Table 3 に示した．プライマー A-03 により増幅されたバンドは，200 bp から 2,000 bp 付近に 10～12 本のバンドが，プライマー F-04 では同様に 9～10 本のバンドが出現した．RAPD パターンはプライマー A-03 では 7 つの型に，プライマー

Table 1. Distribution of contamination by *Staphylococcus aureus* during cooking.

Items	Sort of experimental sources**	Number of *S. aureus* sources detected	Number of strains obtained
Participants	Hands; 26 (Before preparation)	4 (2A*, 2B*, 4C*, 8B*)	24
	Hands; 26 (During preparation)	3 (2A*, 7B*, 8B*)	15
Foods	Foodstuffs; 28	2 (2 Chickens)	5
	Prepared food (salad); 26	1 (prepared by 8B*)	7
Equipments	Frying pans; 2	1 (Used by anyone)	4
	Faucets; 2	1 (Used by anyone)	5
Utensils	Knives; 26	2 (Used by 8B*, 7A*)	3
	Chopsticks for cooking; 26	1 (Used by 6C*)	1
	Total; 162	15	64

* Identified participants.

** The number shown after the semicolon indicates the number of each source.
Samples were taken from the hands of the 26 participants before and during food preparation activities, and from 28 types of foodstuffs, 26 prepared foods, 4 pieces of equipment, and 52 utensils. Out of the 162 specimens, 64 different strains of *S. aureus* were detected.

Table 2. A-03 Primer RAPD patterns of 64 *S. aureus*[a] isolates

PAPD-A Types[b]	Representative example of RAPD Patterns (kb)	strain code[c]	n[d]
a 1		K7、K8、K9	3
a 2		K26、K58	2
a 3		K24,25,27	3
a 4		K51	1
a 5		K29、K30、K31、K33、K34、K35、K36、K37、K38、K39、K40、K41、K42、K43、K44、K45、K46、K53、K54、K55、K56、K57、K59、K60、K61、K63、K65、K66、K68、K71、K72	31
a 6		K3、K4、K5、K6、K10、K11、K12、K13、K14、K15、K16、K17、K18、K19、K20、K21、K22、K23	18
a 7		K73、K74、K75、K76、K77、K80	6

[a] Refer to Table 1.
[b] Classified using A-03 primer.
[c] Refer to Table 4.
[d] Number of strains.

Table 3. F-04 Primer RAPD patterns of 64 *S. aureus*[a] isolates

PAPD-F Types[b]	Representative example of RAPD Patterns (kb)	strain code[c]	n[d]
f 1		K7、K8、K9	3
f 2		K26	1
f 3		K24、K25、K27	3
f 4		K51、K58、K63、K65	4
f 5		K53、K54、K55、K56、K57、K59、K60、K61	8
f 6		K66,68	2
f 7		K29、K30、K31、K33、K34、K35、K36、K37、K38、K39、K40、K41、K42、K43、K44、K45、K46、K71、K72、K73、K74、K75、K76、K77、K80	25
f 8		K3、K4、K5、K6、K10、K11、K12、K13、K14、K15、K16、K17、K18、K19、K20、K21、K22、K23	18

[a] Refer to Table 1.
[b] Classified using A-04 primer.
[c] Refer to Table 4.
[d] Number of strains.

F-04 では 8 つの型に分けられた. *S. aureus* 64 株は, これら 2 種類のプライマーによる RAPD パターンから 11 型 (r1～r11) に別れた (Table 4).

3. BSFGE パターンと多型解析

BSFGE 法による解析は, RAPD 法と同様に *S. aureus* 64 株について行った. 染色体 DNA は 10～12 本に切断され, これらはいずれも 50 kb から 600 kb 付近にバンド形成を示した. 後述の PFGE と比較するため *S. aureus* 39 株の BSFGE パターンを Fig. 1 に示した. これらのパターンは b1～b12 の 12 型に分けられた (Table 4).

4. PFGE パターンと多型解析

無作為に抽出した *S. aureus* 39 株について PFGE 法解析を行った結果, 染色体 DNA は 12～13 本に切断され, これらはいずれも 30 から 700 kb 付近にバンドを形成した (Fig. 2). これらの PFGE パターンは p1～p12 の 12 型に分けられた. BSFGE 法で 100 kb から 200 kb 付近に明瞭に形成されたバンドは, PFGE 法では十分分離されなかった. 一方, 250 kb から 600 kb 付近に形成された 4～5 本のバンドは PFGE 法では明瞭に分離された. PFGE 法で p1～p12 型に分けられた菌株は, BSFGE 法でも b1～b12 型に分けられ各菌株と完全に一致した (Table 4).

Table 4. Origins and classified genotypes of 64 isolates of *S. aureus*

Origin of isolates	Strain code	*n*	RAPD types			BSFGE types	PFGE types
			A-03 primer	F-04 primer	A-03+F-04		
Hand of participant 4C (Before preparation)	K7–9	3	a1	f1	r1	b1	p1
Knife (Used by 7A)	K26	1	a2	f2	r2	b2	p2
Hand of participant 7B (During preparation)	K24, 25, 27	3	a3	f3	r3	b3	p3
Chopsticks (Used by 6C)	K51	1	a4	f4	r4	b4	p4
Frying pan	K53–56	4	a5	f5	r5	b5	p5
Water faucet	K57, 59–61	4					
	K58	1	a2	f4	r6	b6	p6
Foodstuff (Chicken 1)	K63, 65	2	a5		r7	b7	p7
	K66, 68	2		f6	r8	b8	p8
Foodstuff (Chicken 2)	K80	1	a7	f7	r9	b9	p9
Hand of participant 2A (Before preparation)	K10–21	12	a6	f8	r10	b10	p10
Hand of participant 2A (During preparation)	K22, 23	2					
Hand of participant 2B (Before preparation)	K3–K6	4				b11	p11
Hand of participant 8B (Before preparation)	K31, 33–36	5	a5	f7	r11	b12	p12
Hand of participant 8B (During preparation)	K37–46	10					
Knife (Used by 8B)	K29, 30	2					
Prepared food (Salad: prepared by 8B)	K71, 72	2					
	K73–77	5	a7		r9		
Total		64	7 types	8 types	11 types	12 types	12 types

Strains analyzed by PFGE were 39 strains out of 64: K3, K5, K6, K7, K10–12, K14, K19, K21–24, K26, K29–31, K35, K37, K38, K40, K42, K44, K46, K51, K53, K57–K59, K61, K63, K65, K66, K71-73, K75, K77, and K80.

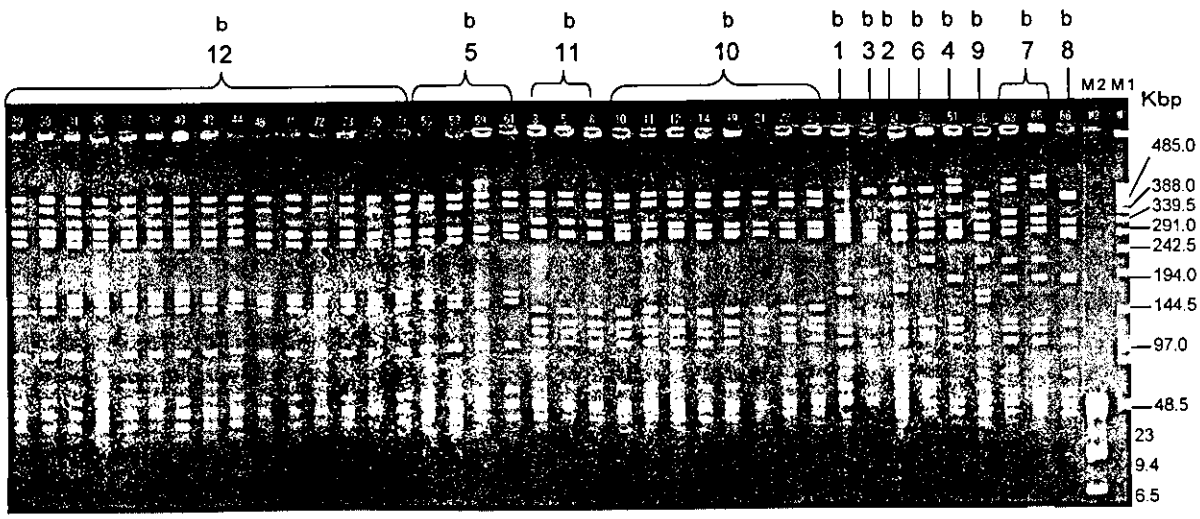

Fig. 1. BSFGE patterns (b1–b12) of selected 39/64 *S. aureus* strains.
Refer to Table 4 for the origins of isolates of each classified pattern.

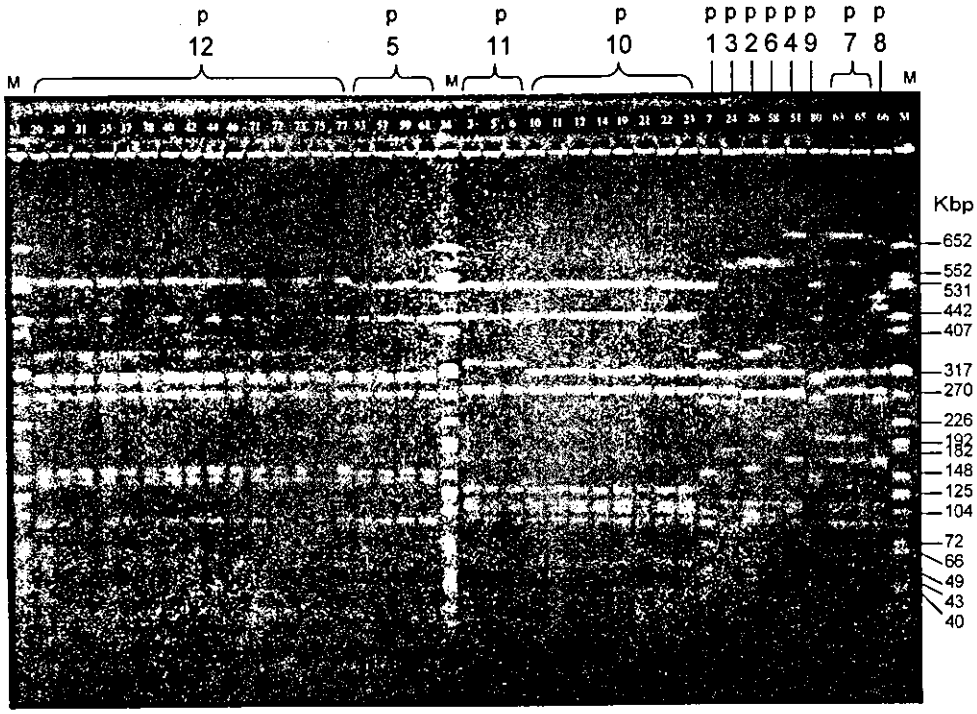

Fig. 2. PFGE patterns (p1–p12) of selected 39/64 *S. aureus* strains.
Refer to Table 4 for the origins of isolates of each classified pattern.

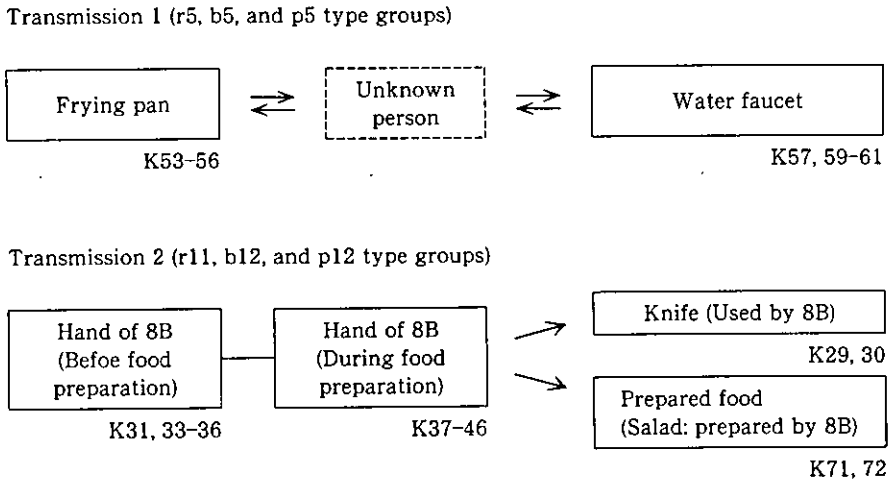

Fig. 3. Proposed transmission patterns of *S. aureus* during cooking.

考　察

Bingen ら[1]はコアグラーゼ陰性のブドウ球菌(CNS)を8名の小児患者から12株分離し，2種のプライマーによるRAPD法で病棟の同区画内の4名は相互に交差感染があり，他区画の4名の患者には交差感染が見られなかったと考察している．本研究においても，RAPD法，BSFGE法およびPFGE法で同一型の菌株が分離されたことから，調理施設内で *S. aureus* の拡散があったと考えられる．

Fig. 3 に示したように，フライパンからの分離菌4株(*S. aureus*; K53～56)とカランからの分離菌の4株（同; K57, 59～61）は同一型を示したことから，特定の実習生の手指から汚染があったと考えられる．また，実習生8Bの手指からの分離菌15株（同; K31, 33～46），包丁からの分離菌2株（同; K29, 30）およびサラダからの分離菌2株（同; K71, 72）はいずれの型別法でも同一型を示したことから，実習生8Bが使用した包丁と，調理したサラダに汚染をしたと考えられる．なお，アトピー性皮膚炎症状を有す者は *S. aureus* を多数保菌していることが示されており[8, 19, 27]，実習生8Bは手指にアトピー性皮膚炎を呈しており，伝播に大きく関与していたと考えられる．

ブドウ球菌食中毒では，汚染源や汚染経路の解析にコ

アグラーゼ型やエンテロトキシン(SE)型別などが広く用いられている．しかし，森田[16]によると食品環境から分離した *S. aureus* の SE 産生率は 37.5% と低く，またこれらの多くは SE 型別不明であることが報告されている．また，PFGE 法では同一コアグラーゼ型別菌株でもさらに細かく型別でき[24]，コアグラーゼ型や SE 型などが同一でも PFGE 型別では異なる型に判定される[25]ことも認められている．それゆえ，本菌の汚染経路などの疫学調査には，PFGE 法などの遺伝子多型解析法が有用であると考えられる．

RAPD 法は PFGE 法に対し再現性および型別能は十分でないと言われている[6,11]．そこで，*Taq*DNA ポリメラーゼの選択や適正なプライマーの検索を行い，さらに複数のプライマーを用いて型別の精度を向上させることが試みられている[23]．藤本ら[5]は *Taq*DNA ポリメラーゼ 5 種類の中で Klen *Taq*DNA polymerase (Sigma) により良好な結果が得られたことを報告している．今回筆者らが用いた AccuPower® PCR PreMix は，Klen *Taq* DNA polymerase と同等の泳動像が得られた．また，使用した 2 つのプライマーは Blanc ら[2]の 12 種類のプライマーの中で 10 本前後のバンドを形成するプライマーを選択した．なお，各菌株については 2～4 回の実験を行い RAPD パターンの再現性を確認した．

RAPD 法で r9 型と r10 型のパターンを示した菌株は各々2 種類の PFGE 型に分けられた．また，r11 型と r9 型に分けられた菌株が PFGE 法による型別では同一パターンを示した．RAPD 法による菌株の型別は PFGE 法による型別と 8 型 (r1～r8 型) は一致したが残りの型は一致しなかった．

*Sma*I 処理による遺伝子多型性は，泳動原理の異なる BSFGE 法と PFGE 法により解析した．BSFGE 法は分画分子量の範囲が 500 bp～3 Mb であり，RFLP (restriction fragment length polymorphism) 解析，酵母染色体の泳動などに活用できる特徴を有しており[14]また，汎用性があり安価に用いる利点がある．これまで BSFGE 法による *S. aureus* の型別報告はないが，Fig. 1 に示すように 100 から 200 kb 付近の分離能が高く，検出バンドも明瞭であり，CHEF 法による PFGE 法と同等またはそれ以上の泳動像が得られた．しかし，250 kb から 600 kb 付近の分離能は PFGE 法に対し，やや劣る傾向が見られた，今後泳動条件などの検討が必要である．

PFGE 法は結果が出るまで数日間を要す．一方，RAPD 法は識別能にやや劣るものの，迅速性に優れ，また簡便に使用できる．食品を取扱う環境から多数の菌株が分離され，これらを短時間に型別などを行い，汚染源を調べる必要がある場合，RAPD 法は有効であると考えられる．なお，われわれは大量調理施設より採取した *S. aureus* の遺伝子多型解析を行い，RAPD 法が汚染源調査の 1 次スクリーニングに有用であることを確認している（投稿準備中）．

要　約

学生の調理実習において，実習生および調理器具，施設から分離した *S. aureus* 64 株について RAPD 法，BSFGE 法および PFGE 法（CHEF 法）による遺伝子多型解析を行い，施設内での本菌の伝播実態を調べた．型別の結果から特定実習生の手指に保有していた *S. aureus* が使用包丁，調理したサラダに拡散していることが推定された．また，カランとフライパンにも伝播が認められた．

RAPD 法による型別では 2 つのプライマーを組み合わせにより 11 型に，BSFGE 法および PFGE 法では 12 型に区別することができた．また，BSFGE 法と PFGE 法型別では両者は完全に一致したが，RAPD 法と PFGE 法による型別で 8 型 (73%) は一致したが残りの型は不一致であった．

RAPD 法は PFGE 法に比べ識別能では劣るが，迅速性および簡便性の特徴を有しており，*S. aureus* の汚染源調査などに十分に活用できると考えられる．

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Characterizations of Adenovirus Type 41 Isolates from Children with Acute Gastroenteritis in Japan, Vietnam, and Korea

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Genetic and antigenic characterizations of 70 strains of adenovirus type 41 (Ad41), isolated between 1998 and 2001 from children in Japan, Vietnam, and Korea, were done by DNA restriction enzyme (RE) analysis, sequencing analysis, and monoclonal antibody (MAb)-based enzyme-linked immunosorbent assay (ELISA). Eight genome types were observed in the present study, among which D25, D26, D27, and D28 were novel genome types. These eight genome types were divided into two genome-type clusters (GTCs) based on phylogenetic analysis of the hypervariable regions (HVRs) of the hexon. GTC1 includes D1, D25, D26, D27, and D28, and the GTC2 contains D4, D12, and D22. The amino acid homologies among the members within a GTC were 97 to 100%, whereas between the members of different GTCs the homologies were 92 to 94%. The specificity of the GTC classification was confirmed by ELISA with MAb 1F, which was selected by the Ad41 prototype Tak strain. It was found that only the isolates of GTC1 but not of GTC2 reacted with MAb 1F. These results suggest that Ad41 isolates from the three countries should be classified into two subtypes. The accumulation of amino acid mutations located in HVRs of hexon are indicative for the classification of Ad41 subtype.

Adenoviruses are responsible for a wide range of disease symptoms. To date, there are 51 recognized serotypes of human adenovirus that are classified into six subgenera (A to F) based on several antigenic, morphological and molecular criteria. Among them, subgenus F, represented by the two serotypes adenovirus type 40 (Ad40) and Ad41, is associated with diarrhea in children; it is found in 1 to 20% of fecal specimens from children with acute gastroenteritis (3, 9, 12, 15, 22, 35). The two serotypes (Ad40 and Ad41) of subgenus F are termed enteric adenovirus (EAd) because of their tropism for the gastrointestinal tract (10, 32). The subgenus F adenoviruses grow poorly in most cell culture systems, in contrast to other cultivable "nonenteric" adenoviruses, and are therefore also termed "noncultivable" or "fastidious" adenovirus (7, 16).

Earlier surveys had shown that the occurrences of Ad40 and Ad41 are approximately equal (6, 11). However, several studies have recently reported a decrease in the rate of isolation of Ad40 and a concomitant increase in the rate of isolation of Ad41 (5, 8, 25, 34). After 1986, Ad41 infection became dominant over Ad40. Our previous studies confirmed that Ad41 was the prevailing serotype of adenovirus associated with acute diarrhea among children also in Japan, Vietnam, and Korea (19). The change in prevalence of Ad41 might have been caused by an antigenic drift, thus increasing the incidence of infection in susceptible individuals (3). Therefore, it is important to characterize these Ad41 strains. In the last decade, there were relatively few comprehensive epidemiological studies of subgenus F adenoviruses from children with diarrhea in Asian countries. The objectives of the present study were (i) to describe the genome types of Ad41 isolates by DNA restriction endonucleases (REs) analysis in Japan, Vietnam, and Korea and (ii) to describe the genetic and antigenic characterization of different genome types of Ad41 isolates in these three countries.

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MATERIALS AND METHODS

Fecal specimens. A total of 3,577 fecal specimens were collected from children between 1 month to 15 years of age with acute diarrhea from Japan, Vietnam, and Korea. These specimens included 1,991 specimens from Tokyo, Osaka, Saga, Maizuru, and Sapporo in Japan collected between July 1998 and June 2001; 1,355 specimens from Ho Chi Minh City in Vietnam collected between December 1999 and November 2000; and 231 specimens from Seoul in Korea collected between January 1998 and July 1999. By using polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (24) and the combination of PCR and restriction fragment length polymorphism methods (31), adenoviruses were detected and serotyped. A total of 158 adenovirus-positive specimens were identified. Of these specimens, 101 specimens were typed as Ad41, the most common serotype, followed by Ad40 (12 isolates), Ad8 (12 isolates), Ad2 (10 isolates), Ad3 (10 isolates), Ad5 (8 isolates), and Ad31 (1 isolate). Four specimens remained untypeable. These Ad41 specimens were used for analysis in the present study.

DNA RE analysis. (i) Virus isolation. Ad41 prototype strain Tak was obtained from the National Institute of Infectious Diseases of Japan. Ad41 specimens were isolated and propagated in Caco-2 cells (human colonic carcinoma cells). The inoculation was performed as previously described (26). The cultured fluids were harvested after infection at the time of maximal cytopathic effect (CPE).

(ii) Extraction of viral DNA. A total of 8 ml of the cultured fluids were centrifuged at 1,353 × *g* for 30 min, and the cells were pelleted. In order to avoid mixing of cellular nucleic acid in the subsequent extraction of viral DNA, ultracentrifugation of the cultured supernatant was performed (40,700 × *g* for 3 h).

TABLE 1. Oligonucleotide primers for PCR sequencing

Primer	Polarity	Sequence (5'-3')	Position[a]
HX5-3	+	cac atc gcc gga cag gat gct tcg gag ta	40–68
HX3-4	−	gtg ttg tga gcc atg ggg aag aag gtg gc	1819–1847
S-29	+	gcc agc acr twc ttt gac at	289–308
S-52	−	ccc atg ttg cca gtg ctg ttg tar tac a	986–1013
S-51	+	ccc aac aga ccc aay tac	937–956
S-53	−	aag ggg ttg acg ttg tcc at	1555–1574

[a] The sequence positions are based on the Ad3 hexon region.

After centrifugation, the supernatant was removed, and the viral pellet was obtained. Subsequent extraction was conducted by a modified Hirt method (44).

(iii) Digestion of DNA by RE. Approximately 1 μg of viral DNA was digested with 10 U of the REs BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, SacI, SmaI, and XhoI (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer's instructions. After digestion, reaction products were loaded onto 1.0% agarose gel containing ethidium bromide (0.5 μg/ml) and run at 50 V for 5 h in Tris-acetate (TAE) buffer. The gel was photographed under UV light. The genome typing system proposed by Adrian et al. (1) was used as the basis for our study. By comparing the resulting patterns with the published restriction profiles of prototype strains, genome type identifications could be achieved.

PCR sequencing analysis of HVRs. In order to understand the genetic relationships between different genome types of Ad41 strains isolated from the three countries in the present study, eight Ad41 representing strains were randomly selected from eight genome types, and their nucleotide sequences of hypervariable regions (HVRs) were determined.

Viral DNA diluted 1:100 was used as a template DNA for PCR. The primers were designed according to the method of Takeuchi et al. (37). In the first PCR, a pair of primers, HX5-3 and HX3-4, was used. The PCR product of 1,800 bp contained all seven HVRs. The second PCR was conducted by using the first PCR product as a template with a combination of the following two pairs of primers: S-29 and S-52 or S-51 and S-53. The primer pairs used are listed in Table 1. The cycling conditions of the PCR were modified in 35 cycles (94°C for 1 min, annealing at 45°C for 2 min, and primer extension at 72°C for 3 min, with a final product extension at 72°C for 7 min). The second PCR products were extracted from 1% agarose gel by using phenol and chloroform. The purified DNA was used to perform enzymatic extension reaction for DNA sequencing with a DNA sequencing kit (BigDye Terminate v3.0 cycle sequencing ready reaction; ABI Prism; Applied Biosystems, Warrington, Great Britain). After the reaction products were cleaned, samples were analyzed by autosequencer (ABI Prism 310 genetic analyzer). Evaluation of all sequence data and analysis of identity were conducted by using DNASIS software (version 1994; Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The sequence alignments of nucleotide and deduced amino acid were carried out by DDBJ (http://www.ddbj.nig.ac.jp) by using the CLUSTAL W program. The phylogenetic tree was constructed with MEGA version 2.1 (18) by using the neighbor-joining method and the bootstrap test (http://www.megasoftware.net/).

Nucleotide sequence accession numbers. Sequence data from the present study were entered in the GenBank/EMBL/DDBJ database under the following accession numbers: Ad41-D1-VN47 (AB103341), Ad41-D4-VN28 (AB103342), Ad41-D12-JP3171 (AB103343), Ad41-D22-Km079 (AB103344), Ad41-D26-JP3106 (AB103345), Ad41-D27-JP2149 (AB103346), Ad41-D25-Ks35 (AB103347), and Ad41-D28-VN1020 (AB103348).

ELISA based on MAb. ELISA based on the use of monoclonal antibodies (MAbs) was utilized to examine the antigen specificity of Ad41 strains. ELISA was performed as described by Nishio et al. (24). Anti-Ad40 rabbit serum was used as capture antibody. The MAbs 15D (adenovirus group-specific), 12D (Ad40 type specific), and 1F (Ad41 type specific) were used as detector antibodies. In each test, cell culture supernatants of Ad41 prototype strain were included as positive controls, and phosphate-buffered saline was used as a negative control. If the optical density value was >0.2, as well as 2-fold greater than the negative control well, the specimen was considered positive.

RESULTS

Genome types of Ad41 isolates from three countries. Seventy Ad41 fecal specimens were isolated successfully, and RE analyses were performed with 10 different REs: BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, SacI, SmaI, and XhoI. To confirm the genome type, restriction patterns were compared to that of the Ad41 prototype Tak strain. The results of enzyme code and genome type determinations obtained in the present study are shown in Table 2. New restriction patterns were observed in cleavage with enzyme EcoRI, KpnI, PstI, SacI, and SmaI. According to the nomenclature system, the new patterns were named as follows: code 7 of EcoRI, code 4 of KpnI, code 4 of PstI, code 3 of SacI, and code 9 of SmaI (Fig. 1a to e).

Analysis of 70 Ad41 isolates from three countries revealed the existence of eight genome types in the present study. Of these genome types, four (D1, D4, D12, and D22) were previously reported by van der Avoort et al. (43). In addition, four novel genome types D25, D26, D27, and D28 are reported here for the first time. The photographs of representative strains of these novel genome types cleaved by the panel endonucleases are shown in Fig. 2. They accounted for 61% (43 of 70) of all Ad41 isolates. The novel genome type D26 had enzyme code 2212141341 and was closely related to another novel genome type D25, differing only in cleavage with BamHI.

In Japan, the novel genome type D26 predominates with 26 isolates, followed by D12 and D27 with 17 and 4 isolates, respectively. In 3 years, D26 and D12 were observed every

TABLE 2. Genome types of Ad41 isolates in Japan, Vietnam, and Korea

Country	Genome type[b]	Enzyme code[a]										Total no. of isolates
		BamHI	BglI	BstEII	EcoRI	HindIII	KpnI	PstI	SacI	SmaI	XhoI	
Japan	**D26**	2	2	1	2	1	**4**	1	**3**	4	2	26
	D12	2	1	2	1	5	1	1	1	2	1	17
	D27	2	2	1	**7**	1	1	1	1	4	2	4
Vietnam	**D28**	2	1	4	4	4	2	**4**	1	**9**	1	10
	D4	3	1	1	1	3	1	1	1	4	1	4
	D1	1	1	1	1	1	1	1	1	1	1	2
Korea	D22	2	1	2	1	5	1	3	1	2	1	4
	D25	1	2	1	2	1	**4**	1	**3**	4	2	1
	D26	2	2	1	2	1	**4**	1	**3**	4	2	2

[a] The boldface numbers indicate a novel pattern.
[b] Novel genome types are indicated in boldface.

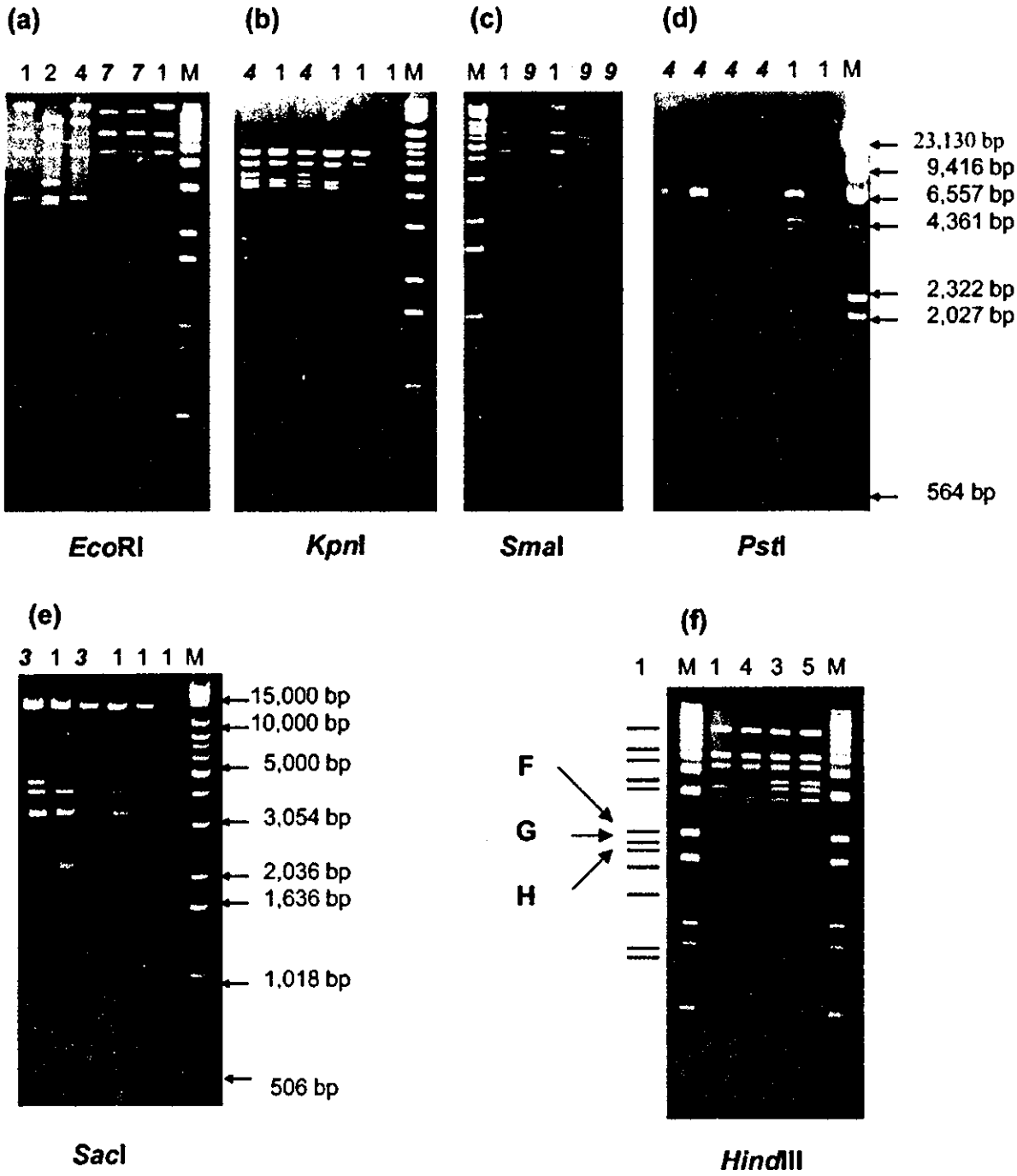

FIG. 1. Novel restriction patterns with EcoRI (a), KpnI (b), PstI (d), SacI (e), and SmaI (c). The numbers above the lanes are the enzyme code; lane M contains molecular weight standards (1-kb DNA extension ladder; Life Technologies), except for lane M of PstI. Lane M of PstI contains molecular weight standards (Marker II, λDNA HindIII digested; TaKaRa). Enzyme code "1" refers to the pattern of the prototype. The boldface italic numbers mark the novel patterns. (f) Restriction patterns with HindIII observed in the present study. The numbers above the lanes are the enzyme code; lane M contains molecular weight standards. The marker lines on the left give the model pattern of enzyme code 1. The arrowed letters F, G, and H refer to the names of the fragments according to the physical map given by Takiff et al. (40). The patterns of codes 3 and 5 lack fragment F.

year. From July 1999 to June 2000, the new genome type D27 appeared, but it was not found the next year. In Vietnam, D28, represented by 10 isolates, was the most common. D4 and D1 were observed in 4 and 2 isolates, respectively. In Korea, D22 predominated with four isolates. Interestingly, the novel genome type D26 was observed in Korea as well. It was present in two isolates, whereas only one isolate of novel genome type D25 was detected.

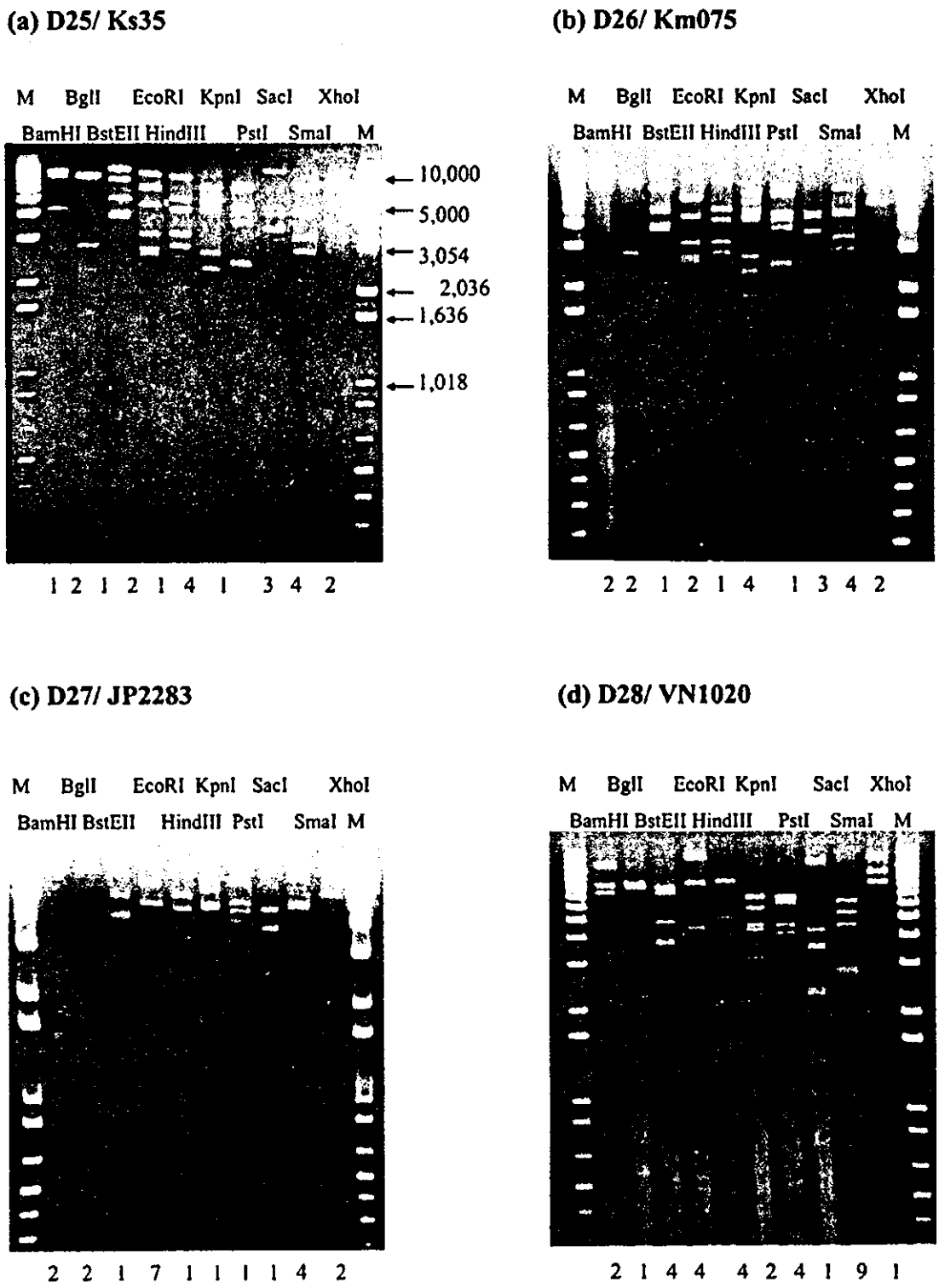

FIG. 2. (a to d) Restriction profiles of novel Ad41 genome type strains isolated from Japan, Vietnam, and Korea, obtained in the present study. (a) D25/Ks35; (b) D26/Km075; (c) D27/JP2283; (d) D28/VN1020. The numbers under the lanes are the enzyme code; lane M contains molecular weight standards.

Genetic analyses of HVRs for eight genome types of Ad41. The HVRs of eight representative strains from eight genome types obtained in the present study were sequenced. The DNA sequences, containing seven hexon HVRs, with a total of 1,232 to 1,235 nucleotides, from the eight strains were determined. Alignments of the predicted amino acid sequences were performed. A phylogenetic tree based on the deduced amino acid sequences of eight representing genome types was subse-

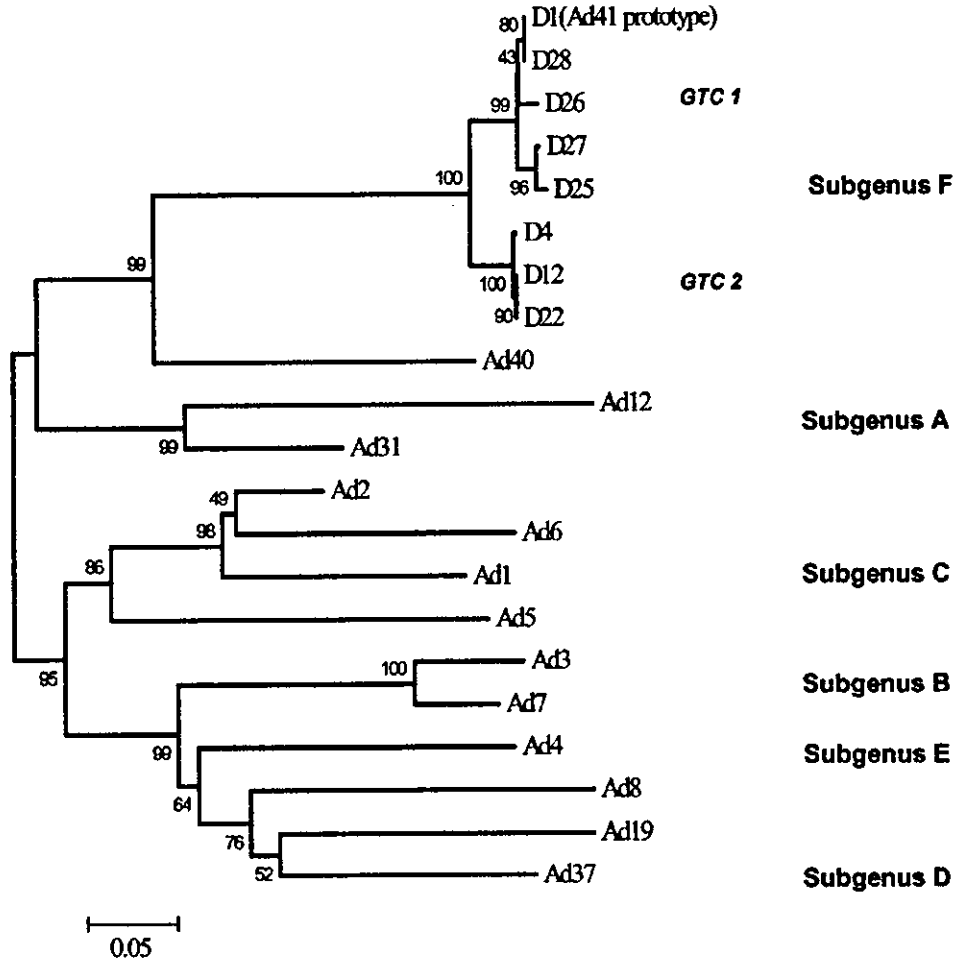

FIG. 3. Phylogenetic tree based on the deduced amino acid sequences of HVRs of Ad reference strains and Ad41 strains isolated from Japan, Vietnam, and Korea. Two GTCs, GTC1 and GTC2, are shown. The sequences of reference strains previously reported are listed in GenBank under the indicated accession numbers: Ad12 (X73487) (36), Ad31 (74661) (29), Ad1 (X67709) (28), Ad2 (J01917) (2), Ad5 (M73260) (17), Ad6 (X67710) (28), Ad3 (X76549) (30), Ad7 (Z48571) (21), Ad4 (X84646) (30), Ad8 (X74663) (29), Ad19 (X98359) (43), Ad37 (X98360) (43), Ad40 (X51782) (42), and Ad41 (X51783) (41).

quently constructed (Fig. 3). It revealed that in the eight genome types representing strains, two genome type clusters (GTCs) are present. GTC1 includes D1 (Ad41 prototype Tak strain), D28, D26, D27, and D25, and GTC2 consists of D4, D12, and D22. The amino acid homology rates in all seven HVRs among the eight representative strains are shown in Table 3. The highest amino acid homologies were observed within a GTC (97 to 100%). Between the members of different GTCs, the homologies were 92 to 94%. The alignments of HVRs predicted amino acid sequences are shown in Fig. 4. The same substitutions in amino acid (aa) 54 (HVR1), aa 74 (HVR2), aa 100 to 101 (HVR3), aa 133 (HVR4), aa 155 to 156, aa 160 (HVR5), aa 190 (HVR6), aa 314 to 316, and aa 323 (HVR7) were presented between GTC1 and GTC2 strains. The members of GTC2 have a common deletion in aa 73 and an insertion in aa 320.

Antigen specificity of Ad41 isolates. MAbs were used to examine the antigen specificity in all Ad41 isolates belonging to eight different genome types. All of these isolates reacted with MAb 15D; none of them reacted with MAb 12D. On the other hand, two antigenic subtypes among eight genome types were distinguished on the basis of the specificity of the MAb 1F selected by Ad41 prototype Tak strain. The isolates that belonged to genome types D1, D28, D26, D27, and D25, were recognized by MAb 1F, but the mem-

TABLE 3. Homology comparison of HVRs between Ad41 isolates and prototype strains Ad40 and Ad41

HVR	% Homology[a] with HVR:								
	Ad40	D1	D26	D27	D28	D25	D4	D12	D22
Ad40 (prototype)		72	72	72	72	72	72	72	72
D1 (Ad41 prototype)	68		98	98	100	98	94	95	95
D26 (JP3106)	68	98		98	98	98	94	94	94
D27 (JP2149)	67	98	99		98	98	94	94	94
D28 (VN1020)	68	100	98	98		98	94	95	95
D25 (Ks35)	67	98	97	99	98		94	94	94
D4 (VN28)	66	94	93	93	94	92		99	99
D12 (JP3171)	66	94	93	93	94	93	99		99
D22 (Km079)	66	94	93	93	94	93	99	100	

[a] The upper right data are the percent homologies of the nucleotide sequences; the lower left data are the percent homologies of the amino acid sequences.

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                    10         20         30         40         50         60         70         80         90        100
D1 (Ad41Tak) ASTYFDIRGV LDRGPSFKPY SGTAYNSLAP KTAPNPCEWK D-NNKIKVRG QAPFIGININ KDNGIQIGTD TTNQPIYADK TYQPEPQVGQ TQWNSEVGAA
D1 (VN47)    ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D26 (JP3106) ********** ********** ********** ********** *-******** ****V****D **K******* ********** ********** **********
D27 (JP2149) ********** ********** ********** ********** *N******** *******S** **K****E** A********* ********** **********
D28 (VN1020) ********** ********** ********** ********** *-******** ********** ********** ********** ********** **********
D25 (Ks35)   ********** ********** ********** ********** *T******** *******S** **K****E** A********* ********** **********
D4 (VN28)    ********** ********** ********** ********** *N******** ***L*****D ********** A*-K****** ********** *********T
D12 (JP3171) ********** ********** ********** ********** *N*-****** ***L*****D ********** A*-K****** ********** *********T
D22 (Km079)  ********** ********** ********** ********** *N*-****** ***L*****D ********** A*-K****** ********** *********T

                                                <      HVR 1        >  <        HVR 2        >  < HVR3

                    110        120        130        140        150        160        170        180        190        200
D1 (Ad41Tak) QKVAGRVLKD TTPMLPCYGS YAKPTNEKGG QASLITNGTD QTLTSDVNLQ FFALPSTPNE PKAVLYAENV SIEAPDTHLV YKPDVAQGTI SSADLLTQQA
D1 (VN47)    ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D26 (JP3106) ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D27 (JP2149) ********** ********** ********** ********** ********** ********** ********** ********** ***G****** **********
D28 (VN1020) ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D25 (Ks35)   ********** ********** ********** ********** ********** ********** ********** ********** ***G****** **********
D4 (VN28)    D********* ********** ********** **N******* ********** ****AN***V ********** T********* *********V **********
D12 (JP3171) D********* ********** ********** **N******* ********** ****AN***V ********** A********* *********V **********
D22 (Km079)  D********* ********** ********** **N******* ********** ****AN***V ********** A********* *********V **********

HVR 3 >                <        HVR 4    >      <      HVR 5      >        < HVR 6>

                    210        220        230        240        250        260        270        280        290        300
D1 (Ad41Tak) APNRPNYIGF RDNFIGLMYY NSTGNMGVLA GQASQLNAVV DLQDRNTELS YQLMLDALGD RSRYFSMWNQ AVDSYDPDVR IIENHGVEDE LPNYCFPLGG
D1 (VN47)    ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D26 (JP3106) ********** ********C* ********** ********** ********** ********** ********** ********** ********** **********
D27 (JP2149) ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D28 (VN1020) ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D25 (Ks35)   ********** ********** ********** ********** ******A*** ********** ********** ********** ********** **********
D4 (VN28)    ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D12 (JP3171) ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D22 (Km079)  ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

                    310        320        330        340        350        360        370        380        390        400
D1 (Ad41Tak) SAATDTYSGI KANGQTWTAD -DNYADRGAE IESGNIFAME INLAANLRRS FLYSNVALYL PDSYKITPDN ITLPENKNTY AYMNGRVAVP SALDTYVNIG
D1 (VN47)    ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
D26 (JP3106) ********** ********** -********* ********** ********** ********** ********** ********** ********** **********
D27 (JP2149) ********** ********** -********* ********** ********** ********** ********** ********** ********** **********
D28 (VN1020) ********** ********** -********* ********** ********** ********** ********** ********** ********** **********
D25 (Ks35)   ********** ********** -********* ********** ********** ********** ********** ********** ********** **********
D4 (VN28)    ********** ***SGN**** N*T******* ********** ********** ********** ********** ********** ********** **********
D12 (JP3171) ********** ***SGN**** N*T******* ********** ********** ********** ********** ********** ********** **********
D22 (Km079)  ********** ***SGN**** N*T******* ********** ********** ********** ********** ********** ********** **********

             <              HVR 7              >

                    410
D1 (Ad41Tak) ARWSPDPMDN VNP
D1 (VN47)    ********** ***
D26 (JP3106) ********** ***
D27 (JP2149) ********** ***
D28 (VN1020) ********** ***
D25 (Ks35)   ********** ***
D4 (VN28)    ********** ***
D12 (JP3171) ********** ***
D22 (Km079)  ********** ***
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FIG. 4. Alignments of the deduced amino acid sequences of hexon, including seven HVRs from eight Ad41 genome type strains. The regions of seven HVRs are given. The borders of the seven HVRs are in accordance with the literature (37). Asterisks indicate the identical residues to the sequence of D1.

bers of D4, D12, and D22 could not be distinguished with the same MAb 1F.

DISCUSSION

In this study, the genome types of Ad41 strains isolated from fecal specimens in Japan, Vietnam, and Korea were determined. In addition, the genetic and antigenic characterizations are given.

EAd are known as fastidious adenoviruses since they do not grow well in conventional cell lines. There are varied reports in the literature concerning the ability of EAd to replicate in different cell lines, such as Chang conjunctival cells, Hep-2 cells, 293 cells, and PLC/PRF/5 cells. The 293 cells, an Ad5-transformed HEK cell line that contains and expresses the Ad5 early region E1A and E1B, were considered useful for the growth of many Ad41 strains (40), but infection yields are still low. Recently, Pinto et al. (27) have reported the use of Caco-2 cell line in isolating laboratory strains of human enteric viruses, including the group A rotavirus type 3, astrovirus serotype 1, and Ad5, Ad40, and Ad41. In their study, for EAd, apparent CPE was observed with laboratory strains, whereas wild-type Ad40 and Ad41 strains failed to induce CPE in Caco-2 cells, though the amplification of viral nucleic acid was confirmed by dot blot hybridization. In our study, Caco-2 cells were used to propagate EAd from fecal specimens. Seventy Ad41 and ten Ad40 (unpublished data) strains from clinical specimens were successfully isolated. The present study confirmed that Caco-2 cells are a useful cell line for research on diarrheal viruses.

Among these genome types observed in Japan, Vietnam, and Korea, four novel genome types (D25, D26, D27, and D28) were observed for the first time, and the other four genome types (D1, D4, D12 and D22) were reported in earlier studies (13, 14, 43). Genome type D12 was present in 17 isolates in Japan over a period of 3 years. This genome type was first isolated in 1982 from The Netherlands and was highly prevalent in the 1980s (43). In Sweden, an identical D12 was also reported, specimens of which were mainly obtained from an outbreak of gastroenteritis in a long-term pediatric ward at a hospital of Stockholm in 1988; it was then found sporadically for several years afterward (13). Another genome type D22 observed in Korea was first isolated during 1984 in The Netherlands, too (43). In the present study, genome type D4 identified in Ho Chi Minh City, Vietnam, was also first found in Malaysia in 1979 (14). These phenomena may indicate that genome types of Ad41 have a scattered global distribution. It is quite possible that the novel genome types observed in the present study are also present in Europe and other continents. Another possibility is that, because of limited research work these novel genome types were not detected.

In studying the profiles of Ad41 isolates obtained with 10 restriction enzymes in three countries, it was found that almost all restriction patterns that appeared in Korean strains were also present in Japanese strains and, in contrast, Vietnamese isolates always exhibited different patterns. For example, Japanese and Korean Ad41 isolates have the patterns 1 and 5 upon cleavage with enzyme HindIII, but Vietnamese strains showed patterns 3 and 4, along with pattern 1. In addition, novel genome type D26 was observed in both Japan and Korea. These data might represent a gradient of antigenic drift reflecting increased travel activities between the two countries.

The occurrences of novel genome types reflect the proceeding mutation of viral DNA. Hexons are important capsid proteins of adenovirus. It was reported that seven HVRs located in hexons take part in the neutralization reaction and contain the epitopes of serotype determination (4). It is interesting to estimate what role these regions might have in identical adenovirus serotypes. Previous studies revealed that hexon contains HVRs that are conserved in different genome types of Ad3 and Ad7 belonging to the subgenus B (20, 38). In the present study, the sequence analyses of HVRs of different genome types of Ad41 isolates from three countries also demonstrated a high level of conservation. The homologies ranged from 92 to 100% and the eight genome types of Ad41 were divided into two clusters according to the predicted amino acid sequences of HVRs. The classification of GTCs had a curious coincidence with the result of ELISA with MAb 1F. It has been confirmed that the MAb 1F may react with hexon protein of Ad41 (24). Therefore, the results presented here suggest that HVRs of hexons may contain epitopes that are associated with the Ad41 subtype.

In comparing the ten enzyme codes, one difference can be found in cleavage with HindIII between GTC1 and GTC2. The members of GTC1 have the HindIII code 1 and 4; the members of GTC2 have codes 3 and 5. Physical map of Ad41 was published by Takiff et al. (39). Tak strain HindIII fragments F and H were determined to lie between map units 51 and 60, which is within the confines of the hexon gene. van der Avoort et al. (43) revealed that D4, D12, and D22 could not react with MAbs 1-23, 3-10, 3-18, and 7-14 and lacked fragment F in common (Fig. 1f). However, in the present study, the nucleotide sequence analysis of hexon-containing HVRs confirmed that the guanine-for-adenine substitution in nucleotide 696, which leads to failure of HindIII-cut viral DNA of GTC2 isolates, was a conservative substitution.

Recently, ELISA with MAbs specifically reacting with Ad40 and Ad41 is widely used for the diagnosis of EAd in fecal specimens. It is a rapid, readily available means for identification of Ad40 and Ad41. MAb 1F was used successfully for screening Ad41 in clinical specimens (24). In the present study, the Ad41 isolates belonging to genome types D4, D12, and D22 did not react with MAb 1F. The application limitation of MAbs was reported in earlier studies (5, 23, 33, 43). Due to virus strain mutation, the MAb may be safely used for screening purposes only temporarily. The genetic variation within one type of adenovirus can evidently affect the diagnosis capability of highly specific MAbs. It has been reported that a commercial MAb-based ELISA could not demonstrate the presence of a highly prevalent genome type of Ad41 in Canada (33). In South Africa, the locally circulating adenoviruses were not detected with a commercial kit utilizing MAbs directed against type-specific epitopes on the adenovirus hexon (23). For Japanese specimens, MAb 1F distinguished all of the Ad41 strains isolated from 1982 to 1988 (24) but failed to react with some Ad41 isolates in 1998 to 2001. These results also confirmed that antigenic drift on the surface of adenovirus occurred frequently. Hexon and fiber compose most of the external capsid of adenovirus. It may be difficult to find other, better targets for reagent antibodies except the hexon and

fiber. The use of pooled MAbs to hexon may provide a practicable way to avoid omissions in detection such as the ones described above when a single MAb was used.

In conclusion, eight genome types of Ad41 were found in Japan, Vietnam, and Korea. Four novel genome types were investigated for the first time in the present study. The existence of two GTCs and the antigenic difference between them may reflect the accumulation of amino acid mutations located in HVRs of the hexon. These amino acid mutations may affect classification of the Ad41 subtype. These data contribute to our understanding of the molecular epidemiological characterization of adenoviruses in these countries. Further studies are needed to obtain additional established strains of the Ad41 genome types in order to clarify the genetic and antigenic relationships between them.

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Evaluation of a Bedside Immunochromatographic Test for Detection of Adenovirus in Respiratory Samples, by Comparison to Virus Isolation, PCR, and Real-Time PCR

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An immunochromatography (IC) kit for human adenovirus (HAdV) was evaluated with 138 patient nasopharyngeal samples. The samples were collected at a sentinel clinic in Japan from January through June 2003. Patients were diagnosed by clinical manifestation of pharyngoconjunctival fever (n = 38) or exudative tonsillitis (n = 100). The IC kit was positive for 84% (116 of 138) of patients diagnosed at bedside. The remaining extract solution of the IC kit test was transferred into maintenance medium and tested via laboratory diagnoses. The IC kit had 95% sensitivity (116 of 122 patients) with HAdV isolation (isolation) as the standard and 91% sensitivity (116 of 128 patients) with PCR as the standard. All of the IC kit-positive samples were isolation and PCR positive. Similarly, all the isolation-positive samples were PCR positive. Twenty-two IC kit-negative samples were evaluated by real-time PCR. Six samples were IC kit negative and isolation positive and contained 3.8×10^7 to 2.5×10^9 copies of the HAdV genome/ml. Five samples that were only PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml, but one sample was real-time PCR negative. We conclude that the IC kit is a useful bedside diagnostic tool for HAdV infections because it has 95% sensitivity (compared to isolation), but a negative result does not always rule out HAdV infection.

Human adenoviruses (HAdVs) are one of the most commonly isolated viruses and are a significant cause of diseases of the respiratory tract and eye. Pharyngoconjunctival fever (PCF) is a widely known adenoviral disease. HAdVs, particularly types 7 and 3, are also known to cause pneumonia (12, 13). Furthermore, HAdVs are increasingly being recognized as fatal pathogens in immunocompromised patients (1, 2, 5, 18).

Recently, four different kinds of diagnostic methods for HAdV infection became available. The virus isolation technique (isolation) is usually considered the "gold standard" but is time-consuming. PCR is a rapid and sensitive diagnostic technique that can be completed in a few hours, and sequencing the amplified products yields useful data. Real-time PCR for HAdVs has recently become available. Real-time PCR produces results quickly, and quantitative data can be obtained (5, 8, 18). In addition to these laboratory diagnostic techniques, immunochromatography (IC) kits have become available for HAdV diagnosis at the patient's bedside (6, 7, 16, 20, 21).

IC kits for HAdVs have been evaluated in comparison with an isolation technique (6, 20), an enzyme-linked immunosorbent assay kit (16), and PCR (7, 21). However, they have not been evaluated in comparison to the combination of isolation, PCR, and real-time PCR. Quantitative evaluation alone was insufficient; therefore, we evaluated an IC kit in comparison to these three methods. The sensitivity and specificity of the IC kit change according to which method is considered to be the gold standard (15).

The purpose of this study was to evaluate the IC kit, qualitatively and quantitatively, in comparison to multiple, sensitive laboratory diagnostic methods. The reliability and limitations of the IC kit were evaluated. The detection limit of the IC kit was evaluated with HAdV type 1, 2, 3, 5, 6, and 7 isolates.

We found that the IC kit had a higher sensitivity than reported previously, although it was less sensitive than isolation, which was less sensitive than PCR and real-time PCR. However, the IC kit was found to have 95% sensitivity compared to HAdV isolation, the diagnostic technique usually considered the gold standard.

MATERIALS AND METHODS

Patients and clinical samples. A total of 138 nasopharyngeal swabs were collected from patients suffering from pharyngoconjunctival fever (n = 38) or exudative tonsillitis (n = 100) during the period of January through June 2003. The mean age of the patients, who ranged in age from <1 to 10 years, was 3.6 ± 0.19 years (mean ± standard deviation [SD]), except for one adult patient who was 38 years old. There was no significant difference in the sex of the patients (72 males and 66 females). All of the patients had temperatures in the range of 38.0 to 40.7°C; the mean temperature was 39.4 ± 0.05°C (mean ± SD). The clinical picture of patients with typical HAdV-associated exudative tonsillitis includes a high fever (>38°C) which persists for about 5 days, and C-reactive protein is usually strongly positive. All clinical samples were collected at the Okafuji Pediatric Clinic, Himeji City, Japan. The protocol was reviewed and approved by the local institutional review board, and informed consent was obtained from all patients.

Clinical diagnosis at bedside. The IC kit used in this study was the SAS Adeno Test (SA Scientific, Inc., San Antonio, Tex.), which was sold under the name Check Ad (AZWELL, Osaka, Japan). The tonsils and posterior pharynx of each patient were vigorously rubbed with a cotton swab moistened in sterile physiological saline. The swabs were extracted with 500 μl of mucolytic agent provided

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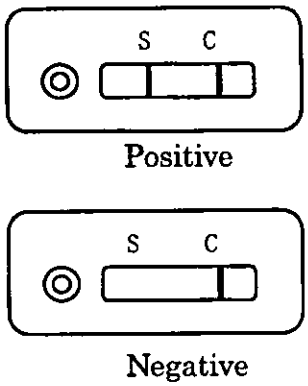

FIG. 1. Detection of adenovirus in a clinical sample by the IC kit. The test is positive if two colored lines appear in the sample (S) and control (C) areas. The test is negative when only one colored line appears in the control area.

by the manufacturer and rubbed in a soft tube. An aliquot of the extract (200 μl) was filtered and dropped into the IC kit device. Both the test tube and filter were provided by the manufacturer. The IC kit indicated an HAdV-positive result when two colored lines appeared in the device. When only one colored line appeared in the control area of the IC kit, the result was HAdV negative (Fig. 1). The remaining extract solution (about 200 μl) was transferred into a test tube containing 2 ml of Dulbecco's modified Eagles's medium (Sigma) and used as a clinical sample for a later laboratory diagnosis. The test tubes were kept at −80°C until use.

Virus isolation and serotyping. Clinical samples were inoculated onto 80% confluent monolayers of HEp-2 cells in duplicate wells of a 24-well plate (Nippon Becton Dickinson, Tokyo, Japan). For cultivation, Dulbecco's modified Eagle's medium (Sigma) supplemented with 2% heat-inactivated fetal calf serum and antibiotics was used as a maintenance medium. The HEp-2 cells were subpassaged three to eight times to allow time for the cytopathic effect (CPE) to develop. When the CPE became evident, the HAdV isolates were serotyped by a neutralization test (NT) by use of antisera purchased from Denkaseiken (Tokyo, Japan). HAdV type 7 (HAdV 7) used in the detection limit test was isolated and identified in 1998 by the same technique. Enteroviruses were isolated and identified in the same way. Isolates which could not be identified by NT using antiserum for HAdV 1 to 7 were identified by a combination of PCR and sequencing techniques.

Viral DNA preparation. HAdV genomes and enterovirus RNAs were prepared directly from the clinical samples with a QIAamp blood kit (QIAGEN) or a High Pure viral nucleic acid kit (Roche Diagnostics). DNAs from HAdV 1, 2, 3, 5, and 7 isolates were extracted with the High Pure kit.

PCR and sequencing. A single-tube multiplex PCR for HAdVs was carried out as reported previously (3). HAdVs were distinguished by the size of the amplified products. HAdVs which amplified to produce 188- and 301-bp DNA fragments were typed as HAdV 3 and non-subgroup B HAdVs, respectively.

Another primer pair for HAdV 3 (and HAdV 7) was designed and used for PCR and sequencing of the hexon region of HAdVs. The sequences of the primers were 5′-AGAATCATGGACTGATACTGATG-3′ (sense) and 5′-AGC CTGTCATTGCCAGGCCAGC-3′ (antisense). Amplification reactions were conducted in 50 μl of reaction mixture containing a 0.5 μM concentration of each of the primers, a 200 μM concentration of each dideoxynucleotide, 1.25 U of *Taq* polymerase (TaKaRa Shuzo, Shiga, Japan), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. The reaction was carried out with a cycle of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and was continued for 35 cycles. In the first cycle, the denaturation step continued for 5 min at 94°C, and in the last cycle, the extension step continued for 5 min at 72°C. The expected product size was 1,596 bp (from position 591 to 2186 based on the hexon sequence of HAdV3; GenBank accession number X76549).

Two RT-PCR methods for enteroviruses developed by Ishiko et al. (11) and Oberste et al. (14) were used for enterovirus identification. PCR-amplified products were sequenced directly as reported previously (3, 4, 17). PCR and RT-PCR were performed using a Thermal Cycler Dice (TaKaRa Shuzo). The nucleotide sequence was determined with a model 310 genetic analyzer (Applied Biosystems Japan [ABI]).

TABLE 1. IC kit and typing results of detected viruses (n = 138)

Virus type(s) identified[a]	No. (%) of patients	No. (%) of patients positive by:			
		IC kit	Virus isolation		Multiplex PCR
			HAdV	Enterovirus	
HAdV 1	11 (8.0)	9 (7.8)	11 (9.0)		11 (8.6)
HAdV 2	17 (12)	17 (15)	17 (14)		17 (13)
HAdV 3	86 (62)	80 (69)	84 (69)		86 (67)
HAdV 5	4 (2.9)	4 (3.4)	4 (3.3)		4 (3.1)
HAdV 6	2 (1.4)	2 (1.7)	2 (1.6)		2 (1.6)
HAdV 3 + 5	1 (0.7)	1 (0.9)	1 (0.8)		1 (0.8)
HAdV 3 + CB 2	2 (1.4)	2 (1.7)	2 (1.6)	2 (33)	2 (1.6)
HAdV 3 + CB 5	1 (0.7)	1 (0.9)	1 (0.8)	1 (17)	1 (0.8)
HAdV NI + CB 2	1 (0.7)			1 (17)	1 (0.8)
HAdV NI	3 (2.2)				3 (2.3)
CB 2	2 (1.4)			2 (33)	
Total	138[b]	116	122	6	128

[a] NI, not identified.
[b] Includes eight patients (5.8% of the total number) negative for all viruses.

Real-time PCR. Real-time PCR for a wide range of HAdVs was used in this study. Primers derived from the highly conserved HAdV hexon 3 and 4 genes by Echavarria et al. (1) were used. The sequences of the primers were 5′-GACAT GACTTTCGAGGTCGATCCCATGGA-3′ (Hex3) and 5′-CCGGCTGAGAA GGGTGTGCGCAGGTA-3′ (Hex4). The expected product size was 140 bp (from position 21589 to 21728 based on the complete sequence of HAdV2; GenBank accession number J01917). One microliter of template DNA was added to a final volume of 25 μl containing 1× SYBR green PCR master mix (ABI) and a 160 nM concentration of the primers Hex3 and Hex4. The real-time PCR was carried out with an ABI PRISM 7900 HT sequence detection system for a cycle of 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min, which was continued for 40 cycles. During thermal cycling, the emissions from each sample were recorded and SDS (sequence detection system) software processed the raw fluorescence data to produce threshold cycle (C_T) values for each sample. The SDS software then computed a standard curve from the C_T value of the diluted standards and extrapolated absolute quantities for the unknown samples based on their C_T values. The DNA of the prototype HAdV 2 was amplified by the PCR system of Echavarria et al. (1) as described above. The product (140 bp) was cloned into a pCR2.1 vector and used as a standard.

Detection limit of the IC kit. HAdV 1, 2, 3, 5, and 6 isolated in this study and HAdV 7 isolated in 1998 were diluted in phosphate-buffered saline and tested with the IC kit. Diluted solutions showing slight but clear positivity by the IC kit were used to determine the detection limits of the IC kit. The detection limits were evaluated by determining the 50% tissue culture infective dose ($TCID_{50}$) per milliliter by use of HEp-2 cells (10) and real-time PCR. Additionally, repeated IC kit detection limit tests were performed with a twofold serially diluted HAdV 3 solution to check the reproducibility. At each concentration, 10 IC kits were tested.

RESULTS

Virus isolation and identification. All the IC kit-positive samples were isolation positive. Six IC kit-negative samples were isolation positive and typed HAdV 1 or 3. Enteroviruses were also isolated individually or as a mixture with HAdVs (Table 1). The IC kit was able to detect HAdVs in coinfected samples. Because enteroviruses grow faster than HAdVs in HEp-2 cells, the CPE of coinfected samples was indistinguishable from that of enterovirus-infected samples. However, the supernatant of coinfected samples was IC kit positive. The IC kit was also useful for confirming HAdV isolation when the HAdV CPE was not clear.

TABLE 2. Results of real-time PCR and typing of the IC kit-negative samples (n = 22)

Test results[a] (n)	Virus type identified in each sample[b]	Copies of genome/ml[c]
PCR pos.; isolation pos. (6)	HAdV 1	3.8×10^7
	HAdV 3	9.0×10^7
	HAdV 3	4.0×10^8
	HAdV 1	4.5×10^8
	HAdV 3	1.3×10^9
	HAdV 3	2.5×10^9
PCR pos.; isolation neg. (6)	HAdV 3	ND
	HAdV 3	3.0×10^4
	HAdV NI	7.1×10^4
	HAdV NI	1.1×10^5
	HAdV NI + CB 2[d]	2.1×10^5
	HAdV NI	3.8×10^5
PCR neg.; isolation neg. (10)	Neg. (n = 9)	ND (n = 9)
	Neg.[e] (n = 1)	3.3×10^5

[a] pos., positive; neg., negative.
[b] NI, not identified.
[c] ND, not detected (the multiplex PCR was positive [188 bp] but the band was weak).
[d] The isolate was neutralized by anti-CB 2 serum.
[e] This sample was recorded as PCR negative because only a very weak band (301 bp) was observed.

PCR, RT-PCR, and real-time PCR. All isolation-positive samples, including IC kit-positive samples, were positive by PCR. However, all HAdV PCR-positive and isolation-negative samples were IC kit negative (Table 1). Samples positive by isolation but IC kit negative had higher concentrations of HAdVs than PCR-positive but isolation-negative samples (Table 2). One isolation-positive sample was coinfected with HAdV (2.1×10^5 copies/ml), and only coxsackievirus type B2 (CB 2) was isolated. Two PCR-amplified products were not clear when stained with ethidium bromide. The two samples did not give concordant results with PCR and real-time PCR. A sample containing HAdV (3.3×10^5 copies/ml) was recorded as PCR negative because only a very weak signal was observed after multiplex PCR. Similarly, one real-time-PCR-negative sample was multiplex PCR positive, but the product band was weak.

IC kit for HAdV. The detection limit of the IC kit was $10^{1.6}$ to $10^{4.0}$ $TCID_{50}$/ml and $10^{4.6}$ to $10^{5.8}$ copies of the HAdV genome/ml. The IC kit was not less sensitive in detecting HAdV 3 and 7 than in detecting HAdV 1, 2, 5, and 6 (Table 3). In repeated IC kit tests, HAdV 3 was positive 10 of 10 times at detection limit concentrations, 3 of 10 times at a twofold serial dilution, and 0 of 10 times at a fourfold serial dilution. A high reproducibility for the IC kit was ascertained.

Five clinical samples that were IC kit and isolation negative but PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml. However, six clinical samples were IC kit negative in spite of the presence of 3.8×10^7 to 2.5×10^9 copies of the HAdV genome/ml. These samples were all isolation positive, and the IC kit had sensitivity to the isolates.

At the bedside, 40 patient samples were recorded as strongly positive, since in each case a positive colored line appeared before the control line appeared. Seventy-three samples were positive within 10 min. Only three samples required 20 min.

TABLE 3. Detection limit of IC kit

Virus type	Detection limit	
	$TCID_{50}$/ml (10^n)	Copies of HAdV genome/ml (10^n)
HAdV 1	2.9	4.6
HAdV 2	3.2	5.4
HAdV 3	1.9	5.8
HAdV 5	3.9	5.8
HAdV 6	4.0	5.3
HAdV 7[a]	1.6	5.6
Mean ± SD (CV)[b]	2.9 ± 1.0 (0.34)	5.4 ± 0.4 (0.08)

[a] This strain was isolated in 1998. Other serotypes were isolated in this study.
[b] CV, coefficient of variation.

DISCUSSION

The IC kit has proven to be a useful method for bedside diagnosis of HAdV infections. HAdV infection is difficult to diagnose by the presence of symptoms alone. The sensitivity of the IC kit was 95% in comparison to HAdV isolation in HEp-2 cells. This percentage is higher than that reported earlier by Tsutsumi et al. (20) (72.6%; 69/95). As stated in the manufacturer's guide for the IC kit, the test is highly dependent on the technique of sample collection. We took a great deal of care in sample collection, transportation, and preservation. Additionally, we had more than 10 years of experience in sampling HAdV for isolation before the IC kit became available, which may explain the high detection level. Hara (7) reported a sensitivity of 94.2% (97 of 103 samples) for the same kit in comparison to isolation and that the high sensitivity is dependent on the method of sample collection.

All IC kit-positive samples were isolation positive. Two enterovirus-infected samples were negative by the IC kit and other HAdV diagnoses; the specificity of the IC kit was 100%, as reported previously (6, 20).

In this study, we were able to identify many HAdV-infected patients within a short period of time because a large outbreak of HAdV occurred in Japan in 2003 (13). This outbreak was the largest of its kind in the previous 10 years. The main causative agent was HAdV 3, which accounted for 69% (84 of 122) of the patients diagnosed by NT. According to previous reports, the IC kit is faster than the enzyme-linked immunosorbent assay kit (which takes at least 40 min) and is more useful for detection of respiratory HAdVs (21). Therefore, the IC kit is the only practical bedside diagnostic tool for respiratory HAdV infections.

The IC kit was useful for detecting HAdV in samples coinfected with HAdV and enterovirus. There was no clinical difference between coinfected patients and other HAdV-infected patients. Therefore, HAdV seemed to be the main causative agent of infection in these patients. Coinfection of HAdV3 and HAdV5 could be identified by PCR-based sequencing (17) and use of the HAdV 3- and 7-specific primers designed in this study.

We found that when the SYBR green real-time PCR method was used with isolated HAdV strains, the detection limit of the IC kit was $10^{4.6}$ to $10^{5.8}$ virus genome copies/ml for HAdV 1, 2, 3, 5, 6, and 7. However, there were clinical samples from which we isolated HAdV 3 (four samples) and HAdV 1 (two samples) that were negative by the IC kit despite concen-

trations of 9.0×10^7 to 2.5×10^9 virus genome copies/ml for HAdV 3 and 3.8×10^7 to 4.5×10^8 virus genome copies/ml for HAdV 1. These clinical samples had virus concentrations within the sensitivity limits of the IC kit; the detection limits for HAdV 3 and HAdV 1 were $10^{5.8}$ and $10^{4.6}$, respectively. According to Hierholzer (9), the bulk of newly synthesized HAdV product is not continuously released but remains cell associated. Only the products that are released into the extraction solution contribute to a positive result by the IC kit. This may explain the 5% false-negative rate.

The IC kit used in this study utilizes a monoclonal antibody made with the HAdV 2 hexon protein. Uchio et al. (21) reported that the IC kit had a lower sensitivity to HAdV 3 and 7 than to HAdV 4, when using serotypes 3, 4, 7, 8, 11, and 37, although these researchers used conjunctival swabs as samples. They reported that the minimum amounts of HAdV 3 and HAdV 8 detected by the IC kit were 4×10^3 and 4×10^4 PFU, respectively. Shimizu et al. (16) reported that the detection limit of an IC kit was $10^{4.45}$ (2.8×10^4) $TCID_{50}$/ml for HAdV 3 and that the sensitivities of other IC kits were similar. In our results, the IC kit was sensitive to $10^{1.9}$ to $10^{4.0}$ $TCID_{50}$/ml for HAdV 1, 2, 3, 5, and 7. Detection limits for the six serotypes were 2.9 ± 1.0 log $TCID_{50}$/ml and 5.4 ± 0.4 log copies of HAdV genome/ml (mean ± SD). The coefficients of variation were 0.34 and 0.08, respectively. Based on the real-time PCR results, the detection limit of the IC kit was approximately $10^{5.4}$ copies of HAdV genome/ml. In repeated IC kit tests, the high reproducibility of the IC kit was confirmed.

In this study period, no clinical samples containing HAdV 7 were obtained. HAdV 7 is known to cause fatal pneumonic infections in children, including nosocomial infections. We confirmed that the detection limits for HAdV 7 were $10^{5.6}$ copies of the HAdV 7 genome/ml and $10^{1.6}$ $TCID_{50}$ of HAdV 7/ml. The sensitivity of the IC kit was not lower for HAdV 7 than for the other serotypes in this study. According to Uchio et al. (21), the detection rate of the IC kit is 31% (8 of 26 patients) for HAdV 3 and 60% (3 of 5 patients) for HAdV 7 compared to that of PCR. We were able to detect the presence of HAdV 3 in respiratory samples with the IC kit 93% of the time (in 80 of 86 samples) in comparison to PCR.

The five samples that were IC kit and isolation negative but multiplex PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml. As isolation requires viable virus, PCR was more sensitive than isolation. It is known that many HAdV particles are not infectious, perhaps because they have genomic defects or lack fiber or some other protein (19).

In conclusion, the IC kit is a useful method for diagnosing HAdV diseases at the bedside because it has 95% sensitivity relative to that of isolation. To prevent nosocomial infections, rapid diagnosis at the bedside is necessary. Furthermore, the IC kit provides pediatricians with information about the prognosis of the respiratory diseases at the patient's first visit. However, because the IC kit test had a 5% false-negative rate compared to isolation, careful interpretation is required in cases where IC kit-negative results are obtained and HAdV infection is suspected.

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A Highly Effective Method for Removing Suspended Poliovirus from Water Using a Positively-Charged Carbon Felt Electrode

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Abstract: **We developed an effective system to eliminate poliovirus from modified tap water using a positively-charged carbon felt electrode. The zeta potential of polioviruses was measured using laser microscopic electrophoresis. Poliovirus adsorption to the electrode was examined by indirect immunofluorescence. The tissue culture infective dose (TCID) of poliovirus type 2 (Sabin strain) was determined using human rhabdomyosarcoma cells (RD cells). Poliovirus VP2 gene copy numbers were assessed by reverse transcription followed by a quantitative real-time polymerase chain reaction. The mean zeta potential of the viruses was −20 mV. Relatively large numbers of polioviruses (10^3 or 4×10^3 $TCID_{50}$/0.1 ml) could be removed by adsorption to the electrode, drastically decreasing TCID and copy numbers of poliovirus genome in the water. Virus elimination was dependent on electric current and time. Thus, the positively-charged carbon felt electrode effectively adsorbed polioviruses. The system may prove applicable to the elimination of certain viruses from water.**

Key words: **Poliovirus, Carbon felt electrode, Zeta potential**

An enormous variety of microorganisms including fungi, bacteria, and viruses exist in our environment (3). Many of these are pathogens causing water-borne infections (25), especially water-borne viruses such as enteroviruses, noroviruses, astroviruses, adenoviruses, rotaviruses, and hepatitis viruses (9, 19, 20). To reduce risk of serious human disease, such viruses need to be eliminated from environmental and drinking water.

Various methods have been developed for removal or inactivation of viruses. Long-standing measures such as heating, particularly boiling, have no effect against heat-stable microorganisms including many spores and viruses (17). While some chemical agents such as alcohols (ethanol or butanol), hypochlorous acid, iodoform, and formaldehyde inactivate various microorganisms, these are ineffective against some agents and are toxic to humans (4). Although filtration methods can remove some bacteria and fungi (21), most viruses pass through the filters. Sobsey et al. have developed an electropositive microporous filtration method (28) that can remove hepatitis A virus, but this filter clogs easily and has a short service life.

In general, surfaces of biologic particles, including those of eukaryotic and prokaryotic cells and viruses in ionic solutions, are negatively charged; this negative charge can be expressed as a zeta potential (12). Accordingly, positively-charged electrodes can adsorb negatively-charged biologic particles such as microorganisms (6). Recently we have developed a new system for eliminating *Bacillus subtilis* spores by electroadsorption using a positively-charged carbon felt disk (7). This system effectively removed spores, and the filter resisted clogging. We refined this system to carry out elimination of water-suspended viruses. In this study, we used poliovirus as a model to assess efficiency of elimination.

Materials and Methods

Modified tap water. The modified tap water used throughout the study as a model of water was deconta-

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Abbreviations: cDNA, complementary DNA; D-MEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein-4-isothiocyanate; PCR, polymerase chain reaction; RD cells, human rhabdomyosarcoma cells; RT-PCR, reverse transcription-PCR; TCID, tissue culture infective dose.