イルス特異的プライマーを用いることで対処すべきであるが，cDNAの種類が増えて煩雑となる場合にはone-step PCRの利用も有効である．

3点目は本法の根幹を成すパンソルビンについてであるが，製品としてメルク社の「PANSORBIN® Cells」が唯一のものである．したがって，品薄や在庫切れ，あるいは将来的な製造中止などによる入手困難な局面もありうるため，その対応策を用意しておく必要がある．プロテインA生産用の黄色ブドウ球菌であるCowan I株（理研・バイオリソースセンターなどから入手可能）を各試験研究機関において液体培地で増やし，ホルマリン固定と熱処理[5]を行うことで相当品を自作できる．回収率などの評価試験では市販品と差がないことも確認している．作ってから1年程度は保存しておけるため，大量にストックしておくことも可能である．

8. おわりに

食品からのウイルス検出法は，10年以上にわたって世界中で検討が重ねられてきているテーマであり，原理の異なるさまざまな手法が考案されている．本稿で紹介したパントラ法は，最初から日常業務に取り入れることを前提として設計されたものであり，ある意味一つの完成形と言える．高速冷却遠心機等の大型機器を使わないことや，必要なすべての試薬を市販品として入手できることは導入が容易であることを意味している．運用面においては，パントラ法は言わば骨格プロトコールであって，抗体などの添加試薬はそれぞれの事情に応じて入れ替えられるように柔軟性を持たせてある．例えば，最初の食品洗滌に用いる緩衝液組成は，モノクローナル抗体の使用も視野に入れたものである（pHとNaClが重要）．また，ガンマグロブリン製剤を使用することで，実際に食品からNoVを検出できた事例もある[8]．今後は実事例を積み重ねながら，随時改良を加えていくことになるが，そのためにもできるだけ多くの機関で使われることを期待している．

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Q ノロウイルス（ノーウォークウイルス）のGII.4 2012変異株

ノロウイルスの変異型が検出されていることについて，その概要を．2006年の流行も遺伝子変異が原因とのことだが，今回の変異型とどのように違うのか．（神奈川県　H）

A ノーウォークウイルスは，毎年流行株が変化する．今シーズン流行している2012年変異株は，2006年流行株と同様，粒子の突起部分のアミノ酸変異が起きており，変異によってヒトの免疫から逃れて流行を起こした

NoVのゲノム構造

ノロウイルスは，ノロウイルス属，*Norwalkvirus*種のウイルスであるから，本来ノーウォークウイルスと呼称される．本稿ではノーウォークウイルス（NoV）として表記する．NoVは，冬期に流行するウイルス性胃腸炎の原因ウイルスの1つであるとともに，食品を媒介とする冬期ウイルス性食中毒の主因ウイルスとして知られている．NoVの潜伏期間は短く，24～48時間である．吐き気，嘔吐，下痢，腹痛などの主症状は2～3日で治まり，後遺症はない．感染者の嘔吐物や便には，1g当たり，10^{6-12}個のウイルスRNAが含まれており，NoV粒子10～100個程度で感染が成立することから，吐物，便の糞口感染により容易に2次感染を引き起こす．非常に早い増殖サイクルを持ち，ヒトからヒトへ個体間を頻繁に渡り歩くため遺伝子の進化速度が速く，ウイルスの抗原性変化が早い．

NoVのゲノムは，全長約7.6kbのプラス一本鎖RNAで，ウイルス複製に関与する非構造蛋白質をコードするORF1，ウイルス粒子を形成する構造蛋白質VP1をコードするORF2，VP2をコードするORF3がある（図1a）．180分子のVP1によって形成されるNoV粒子表面の微細な突起（図1b）は，プロトゥルーディングドメイン（P-domain）に相当する．P-domainのアミノ酸配列が多様性に富むため，NoVは多様な抗原性を示す．

NoVのgenogroupと抗原性

NoVには，genogroup I～Vまで（GI～GV）5つのgenogroupがある．Genogroup GI，GII，GIVがヒトに感染する．GIには14種類，GIIには21種類のそれぞれ抗原性の異なるgenotypeが報告されている．2005年以降，NoVの流行の主流はGII.4である．NoVは，毎年新しい変異株が出現するとともに，全流行株に占めるGII.4の割合が変化する[1]．2005/6シーズンの2004変異株は44%，2006/7シーズンに出現した2006b変異株は，大流行して91%に達した．その後も2006bから変化した子孫ウイルスに相当するGII.4変異株が毎年出現したが，ヒト集団に蓄積した2006b変異株に対する免疫により漸減し，2010/11シーズンには34%にまで縮小した．しかし，2011/12シーズンには，2012変異株が北海道で検出され，58%にまで勢力を拡大した．その後，6～9月にオーストラリアで大流行を起こし，日本（新潟で最初に流行が検出された），欧州，米国に感染が広がった．

現時点での2012/13シーズンのまとめでは，再びGII.4が90%に到達している[2]．2006年から現在に至るNoVゲノム上に生じた遺伝子変異の変遷を追跡すると，P-domainのアミノ酸配列が高速に変化していた[3]．GII.4

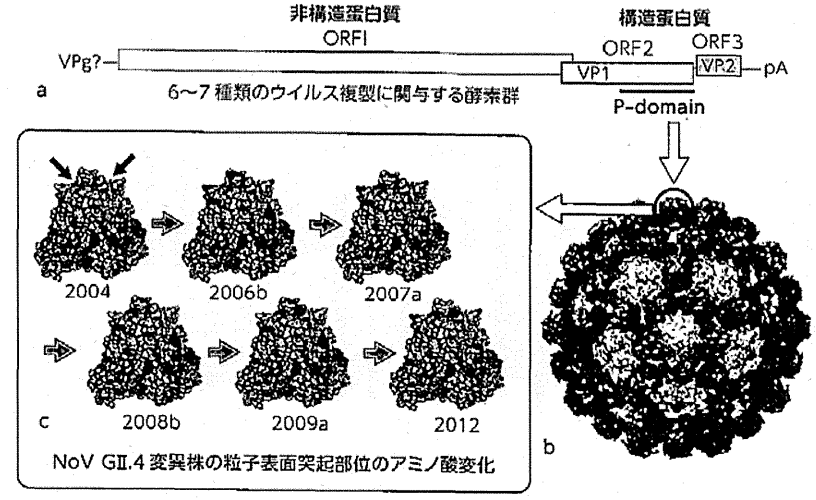

図1　NoVの遺伝子構造と粒子表面のアミノ酸変化による抗原性変化

a：NoVプラス一本鎖RNAゲノムの構造を示した．ORF2/VP1コード領域の下線部分がウイルス粒子表面の突起部分を形成するP-domain．

b：クライオ電子顕微鏡によって解明されたNoV GIIの立体構造（生理学研究所脳機能計測・支援センター形態情報解析室・村田和義准教授提供）．丸印はP-domainを示す．

c：P-domainの拡大図とアミノ酸変異の変遷．2004年変異株を基準に，アミノ酸変異が検出された部分を濃色で示す（アミノ酸配列情報は新潟県環境保健科学研究所調査研究室ウイルス科・田村務科長提供，P-domainへのマッピングは国立感染症研究所病原体ゲノム解析研究センター・横山勝主任研究員提供）．緑色の矢印は，腸管表面の粘膜に存在する糖鎖結合部分を示す．

2004変異株を基準に今シーズンの2012変異株に至るまでP-domain上のアミノ酸変異を調べると，大流行を起こした2006年と2012年に糖鎖結合部位近傍にアミノ酸変化があることが明らかになった．2006b変異株の子孫である2007～2009，2010変異株は2006b変異株が獲得した変異をそのまま維持しており，2006年変異株に対するヒト集団内の集団免疫の蓄積により，徐々に勢力を奪われ漸減したと考えられた[3)4)]．しかし，2011/12シーズンに突然出現した2012変異株は，2006変異株の直接の子孫に当たらず，糖鎖結合部位近傍を2006年株とは異なるアミノ酸に変化させていた．これが集団免疫からの逃避につながり，再びGII.4の世界的大流行をもたらしたと考えられる．

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◆回　答

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資　料

Fast PCR酵素を用いた蛍光RT-マルチプレックスPCR法による下痢症ウイルスの迅速検出

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Rapid Detection of Diarrheal Viruses using a Reverse Transcription Fluorescent Multiplex PCR Assay with Fast PCR Enzyme

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蛍光RT-マルチプレックスPCR法における下痢症ウイルス４種（ノロウイルスＧⅠ，ノロウイルスＧⅡ，サポウイルス，アストロウイルス）の迅速検出を図るため，Fast PCR用の酵素の適用とPCR反応時間の短縮について検討した．Fast PCR用酵素の使用により，これまでの半分以下の反応時間（1.5時間）で，同程度の検出限界を維持したまま４種の対象ウイルスを検出できることが確認された．

Key words：Fast PCR，蛍光RT-マルチプレックスPCR法，時間短縮，下痢症ウイルス

緒　　言

下痢症の起因ウイルスは種々知られており［1-4］，それぞれを個別に検査すると労力，コスト面ともに大きな負担となる．一方，マルチプレックスPCR法は複数の対象を網羅的に検出できることから，下痢症ウイルスについても多くの検査系が報告されている［5-11］．さらに我々は，マルチプレックスPCR法の発展系として，蛍光標識プライマーを用いることにより増幅産物の色とサイズで下痢症ウイルス４種の識別ができる蛍光RT-マルチプレックスPCR法について報告している［12］．最近の情勢としてはPCR反応時間を大幅に短縮できるFast PCR用の酵素が各メーカーから販売されており，検査時間の迅速化が図られつつある．

そこで我々は検査時間の短縮を目的に，下痢症ウイルス４種（ノロウイルスＧⅠ，ノロウイルスＧⅡ，サポウイルス，アストロウイルス）の蛍光RT-マルチプレックスPCR法にFast PCR用の酵素の適用を試み，これまでの方法との比較を行った．

材料および方法

1　供試サンプル

既知検体として食中毒疑い，感染性胃腸炎集団発生28事例，75検体（2010/11年シーズン16事例，39検体及び2010/11年シーズン以前12事例，36検体）を用いた．

2　RNA抽出と逆転写反応条件

10%糞便乳剤からQIAamp Viral RNA mini Kit（キアゲン）によりRNA抽出を行った．抽出したRNAは使用まで－80℃で保管した．

逆転写反応は5×buffer 4μl，10mM dNTPs 4μl，50μM Random primer pd(N)$_9$（タカラバイオ）1μl，RNase inhibitor（40U/μl）（TOYOBO）0.5μl，ReverTra Ace（100 U/μl）（TOYOBO）1μlを含む反応液に抽出RNA9.5μlを加え，30℃・10分，42℃・60分，99℃・5分の条件で行った．

3　マルチプレックスPCR反応条件

Fast PCR酵素としてマルチプレックスPCR反応にAmpliTaq Gold Fast PCR Master Mix, UP（Life Technologies）を用い，表1に示す4色のAlexa蛍光［13］

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で標識したプライマー［3，7，14，15］を所定の終濃度（濃度Ⅰ～Ⅲ）になるよう加えた反応液18μlにcDNAを2μl加え，95℃・10分の熱変性後，96℃・3秒，57℃・3～30秒，68℃・5秒を40サイクル行い，最後に72℃・10分の最終伸長を行った．比較対照には従来のPCR酵素として，Multiplex PCR Assay Kit（タカラバイオ）を用い，表1に示すプライマー（濃度Ⅲ）を含む反応液48μlにcDNAを2μl加え，94℃・1分の熱変性後，94℃・30秒，57℃・90秒，72℃・90秒を40サイクル行い，最後に72℃・10分の最終伸長を行った．

表1　蛍光RT-マルチプレックスPCRに用いるプライマーと反応液中の濃度

対象ウイルス	プライマー名	標識蛍光	引　用	反応液中のプライマー濃度（μM）		
				Ⅰ	Ⅱ	Ⅲ
Norovirus GⅠ	G1SKF	Alexa Fluor 488	Kojima et al. (2002)	0.4	0.2	0.4
	G1SKR			0.4	0.2	0.4
Norovirus GⅡ	G2SKF	Alexa Fluor 594	Kojima et al. (2002)	0.4	0.2	0.4
	G2SKR			0.4	0.2	0.4
	G2ALSKR		Nishida et al. (2007)	0.4	0.2	0.2
Sapovirus	SV-F21	Alexa Fluor 532	Okada et al. (2002)	0.4	0.2	0.2
	SV-R2			0.4	0.2	0.2
Astrovirus	AC1'	Alexa Fluor 350	Sakon et al. (2000)	0.4	0.2	0.2
	AC230			0.4	0.2	0.2

4　電気泳動方法

電気泳動には2％アガロースゲルを用い，マルチプレックスPCR反応液10μlをアプライした．サイズマーカーには100bpラダー（GEヘルスケア）に1μlのEzVISION（Amresco）を混合したものを使用した．電気泳動後にUVトランスイルミネーター上でUV（312nm）を照射して増幅産物の蛍光バンドを観察後，エチジウムブロマイド染色して再度確認した．

5　検出限界の比較

検出限界の比較は，リアルタイムPCR法でウイルスコピー数定量済みの陽性検体由来cDNAをEASY Dilution（タカラバイオ）で希釈した10倍段階希釈シリーズをテンプレートとして用いた．

結　　果

1　アニーリング時間の検討

各ウイルスの陽性検体由来cDNAの10倍段階希釈シリーズをテンプレートとして，アニーリング時間を変えて蛍光RT-マルチプレックスPCRを実施後，電気泳動，エチジウムブロマイド染色により増幅産物を確認した．ノロウイルスGⅠおよびGⅡのcDNAの10倍段階希釈シリーズについてアニーリング時間を3，6，10，20，30秒で比較したところ，時間が長くなるほどバンドが濃くなり，検出限界が向上する傾向が認められた（図1）．時間短縮の観点からアニーリング時間3秒と10秒について，サポウイルスとアストロウイルスの陽性検体由来cDNAの10倍希釈段階シリーズで比較したところ，アニーリング時間10秒でバンドが濃く，一部の検体で検出限界が向上した（図1）．

2　最適プライマー濃度の検討

マルチプレックスPCR反応のアニーリング時間を10秒とし，プライマー濃度を各0.4μM（濃度Ⅰ），各0.2μM（濃度Ⅱ），アストロウイルス，サポウイルスのプライマー及びノロウイルスGⅡのG2ALSKRのみを0.2μMでその他を0.4μM（濃度Ⅲ）にした条件で比較した（表2）．濃度Ⅱでは，濃度Ⅰに比べノロウイルスGⅡおよびサポウイルスの検出限界コピー数が1オーダー上がった．一方，濃度Ⅲでは，ノロウイルスGⅠおよびGⅡの検出限界コピー数が1オーダー下がった．希釈前のcDNA中に含まれるウイルスゲノムコピー数から各ウイルスの検出限界値を推定すると，濃度Ⅰで10^2～10^3コピー/反応，濃度Ⅱで10^2～10^4コピー/反応，濃度Ⅲで10^2～10^3コピー/反応であった．比較対照としてMultiplex PCR Assay Kit（濃度Ⅲ）での蛍光RT-マルチプレックスPCR法の検出限界は，10^1～10^3コピー/反応であった（表2）．

3　Fast PCR酵素を用いた蛍光RT-マルチプレックスPCR法による検出結果と評価

食中毒疑いまたは感染性胃腸炎集団発生事例の28事例（75検体）を用い，AmpliTaq Gold Fast PCR Master Mix, UPとこれまで用いてきたMultiplex PCR Assay Kitで蛍光RT-マルチプレックスPCR法でウイルス検出を行い，結果を比較した．検体毎の検出結果の比較では，ノロウイルスGⅠ及びGⅡの重感染1検体においてMultiplex

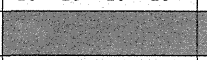
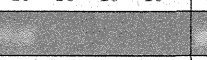
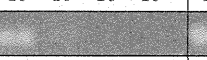
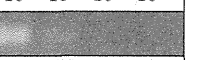
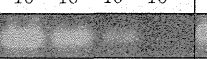
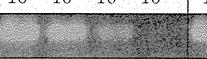
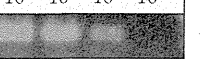
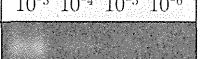
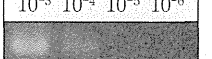
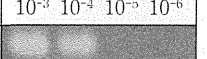
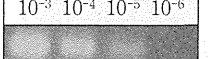
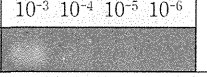
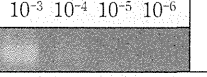
対象ウイルス	検体番号	アニーリング時間				
		3秒	6秒	10秒	20秒	30秒
Norovirus G Ⅰ	希釈率	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}
	1522					
Norovirus G Ⅱ	希釈率	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}	10^{-1} 10^{-2} 10^{-3} 10^{-4}
	1411					
	希釈率	10^{-1} 10^{-2} 10^{-3} 10^{-4}		10^{-1} 10^{-2} 10^{-3} 10^{-4}		
	1294					
Sapovirus	希釈率	10^{-3} 10^{-4} 10^{-5} 10^{-6}		10^{-3} 10^{-4} 10^{-5} 10^{-6}		
	790					
	希釈率	10^{-3} 10^{-4} 10^{-5} 10^{-6}		10^{-3} 10^{-4} 10^{-5} 10^{-6}		
	792					
Astrovirus	希釈率	10^{-3} 10^{-4} 10^{-5} 10^{-6}		10^{-3} 10^{-4} 10^{-5} 10^{-6}		
	1387					
	希釈率	10^{-3} 10^{-4} 10^{-5} 10^{-6}		10^{-3} 10^{-4} 10^{-5} 10^{-6}		
	1388					

図1　Fast PCR酵素を用いた蛍光RT-マルチプレックスPCR反応時のアニーリング時間と検出限界

表2　Fast PCR酵素を用いた蛍光RT-マルチプレックスPCRのプライマー濃度と検出限界

対象ウイルス	検体No.	ウイルス濃度（copy/μl）	試　薬	プライマー濃度	検体希釈率						検出限界反応液中のコピー数
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
Norovirus G Ⅰ	1522	3.0×10^{4}	AmpliTaq Gold Fast PCR	Ⅰ	＋	－	－	－			6.0×10^{3}
				Ⅱ	（＋）	－	－	－			6.0×10^{3}
				Ⅲ	＋	（＋）	－	－			6.0×10^{2}
			Multiplex PCR Assay Kit	Ⅲ		＋	－	－	－		6.0×10^{2}
Norovirus G Ⅱ	1411	1.8×10^{6}	AmpliTaq Gold Fast PCR	Ⅰ		＋	（＋）	－	－		3.6×10^{3}
				Ⅱ		＋	（＋）	－	－		3.6×10^{3}
				Ⅲ		＋	（＋）	（＋）	－		3.6×10^{2}
			Multiplex PCR Assay Kit	Ⅲ		＋	（＋）	－	－		3.6×10^{3}
	1294	6.5×10^{4}	AmpliTaq Gold Fast PCR	Ⅰ	＋	（＋）	－	－			1.3×10^{3}
				Ⅱ	＋	－	－	－			1.3×10^{4}
				Ⅲ	＋	（＋）	－	－			1.3×10^{3}
			Multiplex PCR Assay Kit	Ⅲ	＋	（＋）	－	－			1.3×10^{3}
Sapovirus	790	4.1×10^{6}	AmpliTaq Gold Fast PCR	Ⅰ			＋	－	－	－	8.2×10^{3}
				Ⅱ			（＋）	－	－	－	8.2×10^{3}
				Ⅲ			（＋）	－	－	－	8.2×10^{3}
			Multiplex PCR Assay Kit	Ⅲ			＋	－	－	－	8.2×10^{3}
	792	2.6×10^{6}	AmpliTaq Gold Fast PCR	Ⅰ			（＋）	－	－	－	5.2×10^{3}
				Ⅱ			（＋）	－	－	－	5.2×10^{3}
				Ⅲ			（＋）	－	－	－	5.2×10^{3}
			Multiplex PCR Assay Kit	Ⅲ			＋	（＋）	－	－	5.2×10^{2}
Astrovirus	1387	1.2×10^{6}	AmpliTaq Gold Fast PCR	Ⅰ			＋	（＋）	－	－	2.4×10^{2}
				Ⅱ			＋	（＋）	－	－	2.4×10^{2}
				Ⅲ			＋	（＋）	－	－	2.4×10^{2}
			Multiplex PCR Assay Kit	Ⅲ			（＋）	－	－	－	2.4×10^{3}
	1388	4.0×10^{4}	AmpliTaq Gold Fast PCR	Ⅰ		（＋）	－	－	－		8.0×10^{2}
				Ⅱ		（＋）	－	－	－		8.0×10^{2}
				Ⅲ		（＋）	－	－	－		8.0×10^{2}
			Multiplex PCR Assay Kit	Ⅲ			＋	－	－	－	8.0×10^{1}

バンドの濃さ：＋明瞭，（＋）弱い，－なし

PCR Assay Kitでは両者が検出されたのに対し，AmpliTaq Gold Fast PCR Master Mix, UPではノロウイルスＧⅠのみが検出された．他の74検体については両者とも同一結果となった（表３）．事例毎での検査結果では両者とも同じ結果になった．PCRの反応時間は，Multiplex PCR Assay Kitが約3.5時間に対し，AmpliTaq Gold Fast PCR Master Mix, UPは1.5時間であった．

表３　Fast PCR酵素を用いた蛍光RT-マルチプレックスPCR法による食中毒・感染症事例検体での検出結果

PCR酵素	検出ウイルス					
	Norovirus ＧⅠ	Norovirus ＧⅡ	Norovirus ＧⅠ&ＧⅡ	Sapovirus	Astrovirus	陰性
検体別（75検体）						
AmpliTaq Gold Fast PCR	6	54	2	3	1	9
Multiplex PCR Assay Kit（対照）	5	54	3	3	1	9
事例別（27事例）						
AmpliTaq Gold Fast PCR	2	18	3	1	1	2
Multiplex PCR Assay Kit（対照）	2	18	3	1	1	2

考　　察

Fast PCR用の酵素を用いた蛍光RT-マルチプレックスPCR法の反応条件として，アニーリング時間について検討した．AmpliTaq Gold Fast PCR Master Mix, UPの使用に際しては，アニーリング時間が３秒に設定されているが，マルチプレックスPCR法では，複数のプライマーが増幅に関与するため，熱変性や伸長反応に比べるとアニーリング時間は重要な要因になると思われる．ノロウイルスＧⅠおよびＧⅡのcDNA10倍段階希釈シリーズを用い，アニーリング時間を３，６，10，20，30秒に設定して反応を行った結果では，いずれも時間が長くなるにつれて検出限界の向上や増幅産物のバンドが濃くなる傾向であった．また，３秒と10秒のみについてノロウイルスＧⅡ，サポウイルスとアストロウイルスのcDNA10倍段階希釈シリーズを用い比較した結果では，３秒に比べ10秒において同様な傾向が認められた．時間短縮の観点からアニーリング時間を長く設定することは時間短縮の目的に反することと，Fast PCRの特性を活かすためにアニーリング時間は10秒での使用が適切であると思われた．

マルチプレックスPCR法においては，それぞれの検出対象に対応したプライマーセットが反応液中に含まれるため，PCR増幅反応中にお互いのプライマーが競合する可能性があり，適正な濃度に設定することが必要である．今回各プライマー0.4μM（濃度Ⅰ）を基本に，濃度Ⅱ，Ⅲで比較したところ，濃度Ⅲでノロウイルスの検出限界が向上した．サポウイルス，アストロウイルスの検出プライマーを半量にすることで，プライマー間の競合作用を抑え，結果としてノロウイルスＧⅠとＧⅡの検出限界が向上したように思われる．Multiplex PCR Assay Kitを使用した場合（濃度Ⅲ）での検出限界と比較した場合，検体毎で比較すると多少の優劣はあるが，両者は同等程度の検出限界であると判断された．

これまでの条件検討の結果から，AmpliTaq Gold Fast PCR Master Mix, UPでの蛍光RT-マルチプレックスPCR反応条件をアニーリング時間10秒，プライマー濃度Ⅲに設定した．本条件で，食中毒疑いまたは感染性胃腸炎集団発生事例の28事例（75検体）を用いてMultiplex PCR Assay Kitでの蛍光RT-マルチプレックスPCRとの結果を比較したところ，ノロウイルスＧⅠ及びＧⅡの重感染１検体の結果を除き，他は同一の結果となった．重感染の場合，増幅効率の高いほうのウイルスが検出されることから，両試薬の特性により各ウイルスの増幅効率に差が生じたものと思われる．特に二枚貝の喫食が関係した食中毒では複数のウイルスが検出されることがあり［16, 17］，マルチプレックスPCR法でこのような事例を検査する場合は留意しなければならない．しかし，事例別での検査結果に違いは認められなかったことから，十分な数の検体数が確保できるのであれば，Fast PCR用の酵素を使用しても問題がないと考えられた．しかしながら，Fast PCRは各ステップの反応時間が短いことから，利用に際しては使用するサーマルサイクラーとの相性を事前に見極め，各検査所で反応条件の適正化を図っておく必要があると思われる．

今回，蛍光RT-マルチプレックスPCR法にFast PCR酵素を用いることで，これまでとほぼ同等の検出限界を維持しながらPCR反応時間を従来の3.5時間から半分以下の1.5時間に短縮することが可能であった．呼吸器系ウイルスでは，Fast PCR用酵素を用いたマルチプレックスnested-PCR法が報告されているが，こちらも同様にFast PCR用酵素を使用することでPCR反応時間が１/５以下に大幅短縮されている［18］．核酸抽出などの検体の前処理，増幅産物の電気泳動時間については現行では改善の余地はそれほどないが，Fast PCR用の酵素を用いることでマルチプレックスPCRの反応時間を短縮でき，検査の迅速化が図れる．このことは，他の検査系についてもFast

PCR用酵素を適用すれば検査時間の短縮が図れることを意味している．特に食中毒や集団感染症事件等の発生時には的確な行政対応，蔓延防止のための迅速な原因究明が求められる．しかしながら広範なエリアを有する自治体では，検体の収集から搬入までに時間を要するため，検体搬入が遅れれば検査結果を即日返すことが難しいケースも生じる．そのような時に今回のようにFast PCR用の酵素をマルチプレックスPCR法に使用することで，労務の軽減，効率化，迅速化が図れる．さらに正確な検査結果を即日返すことができれば，行政対応の面からも非常に有用であると言える．

結　　語

蛍光RT-マルチプレックスPCR法の反応酵素にFast PCR用の酵素を用いることで，反応時間を半分以下（1.5時間）に短縮することが可能になった．また，各ウイルスの検出限界は従来のマルチプレックス用PCR酵素を用いた場合とほぼ同等であった．今後，食中毒疑い，感染性胃腸炎集団発生事例の検査の迅速化が期待できる．

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Detection of Sapoviruses and Noroviruses in an Outbreak of Gastroenteritis Linked Genetically to Shellfish

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Norovirus (NoV) and sapovirus (SaV) are important pathogens of human gastroenteritis. Compared to NoV, the transmission route of SaV is unclear. An outbreak of gastroenteritis occurred at a restaurant in June 2008, and SaV and NoV were detected in fecal specimens from 17 people who ate at the restaurant and one asymptomatic food handler and also in stripped shellfish and liquids remaining in the shellfish packages by reverse transcription-polymerase chain reaction (RT-PCR) and/or real-time RT-PCR. Nucleotide sequencing analysis of the RT-PCR products corresponding to the partial capsid region revealed 99.3–100% identities for SaV and 98.6–99.3% identities for NoV among the digestive diverticulum of the frozen stripped shellfish (*Ruditapes philippinarum*), "Asari," the package liquid, and feces from symptomatic or asymptomatic guests. These results suggested a link between the consumption of contaminated shellfish and clinical features in the patients. While the transmission of NoV by shellfish has been reported, this report shows that SaV can also be transmitted by shellfish. ***J. Med. Virol. 82:1247–1254, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: norovirus; sapovirus; shellfish; gastroenteritis; phylogenetic analysis

INTRODUCTION

Norovirus (NoV) and sapovirus (SaV) are important viral causes of human gastroenteritis. NoV and SaV are non-enveloped viruses, with positive-sense, single-strand RNA molecules. NoV and SaV are composed of many genetically distinct strains [Farkas et al., 2004; Zheng et al., 2006]. NoV can be divided into five major genogroups, GI, GII, GIII, GIV, and GV, of which GI, GII, and GIV have been detected in humans. SaV can be divided into at least five major genogroups, GI, GII, GIII, GIV, and GV, of which GI, GII, GIV, and GV have been detected in humans. NoV and SaV cannot be propagated in cell culture. Therefore, reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR have been used widely for detection of these viruses in clinical and environmental specimens [Harada et al., 2009; Iwai et al., 2009]. Nucleotide sequencing is also used widely for molecular characterization of NoVs and SaVs from clinical specimens.

A number of outbreaks of gastroenteritis associated with NoV or SaV have been reported [Schvoerer et al., 1999; Cheesbrough et al., 2000; Inouye et al., 2000; Kageyama et al., 2004; Bon et al., 2005; Johansson et al., 2005; Blanton et al., 2006; Gallay et al., 2006; Wu et al., 2006; Svraka et al., 2007; Shinkawa et al., 2008; Wu et al., 2008; Pang et al., 2009]. NoV and SaV have been detected by RT-PCR in fecal samples from both symptomatic and asymptomatic persons [Ozawa et al., 2007; Motomura et al., 2008; Iwakiri et al., 2009; Yoshida et al., 2009], vomit samples of infected persons [Chadwick and McCann, 1994; Marks et al., 2000], environmental water [Ueki et al., 2005; Hansman et al., 2007c; Aw et al., 2009], and shellfish [Nishida et al., 2003; Costantini et al., 2006; Hansman et al., 2007a; Hansman et al., 2008]. Food-borne outbreaks of acute gastroenteritis associated with NoV and SaV have been reported [Noel et al., 1997; Girish et al., 2002; Johansson

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et al., 2002; Le Guyader et al., 2004; Friedman et al., 2005; Gallimore et al., 2005; Usuku et al., 2008]. However, there are some reports showing a direct link between patients and suspected food as vehicles for NoV gastroenteritis [Daniels et al., 2000; Kobayashi et al., 2004; Nenonen et al., 2009] and there are no such reports for SaV gastroenteritis.

The aims of this study were to test for NoV and SaV in stool specimens collected from subjects in a shellfish-associated outbreak of gastroenteritis and from the same batch of suspected shellfish as food vehicles and to conduct genetic characterization of any NoV and SaV strains. Nucleotide sequencing of the RT-PCR products corresponding to the partial capsid gene revealed the presence of very similar or identical viral nucleotide sequences in the fecal specimens from the patients and the suspected shellfish.

MATERIALS AND METHODS

Fecal Specimens

Fecal specimens were collected from 23 restaurant guests with symptoms of gastroenteritis and an additional person in 3 groups (A, B, and D) and from 10 asymptomatic food handlers. The specimens were stored at −80°C until use.

Shellfish Samples

Frozen stripped shellfish prepared from *Ruditapes philippinarum* and the liquids from shellfish (package liquid) remaining in three 1-kg packages of the shellfish were tested after storage at −80°C. The shellfish and package liquids were collected from the same batch as that used for the suspected menu item (undercooked clam au gratin).

RNA Extraction and cDNA Synthesis

Nucleic acids were extracted from 100 μl of a 10% (w/v) stool suspension with an SV total RNA Isolation System (Promega, Tokyo, Japan) according to the manufacturer's protocol. The digestive diverticulum of the stripped shellfish (designated as Asari-DT) was prepared using a previously described method [Ueki et al., 2005] with some modifications. Briefly, the digestive diverticula of 20 stripped shellfish were removed by dissection and 1 g of the digestive diverticula was homogenized with an Eppendorf micropestle (Eppendorf, Tokyo, Japan) in 1 ml of phosphate-buffered saline without magnesium and calcium (PBS [−]); then, 25 mg of α-amylase (Wako Pure Chemical Industries, Osaka, Japan) was added, and the mixture was incubated at 37°C for 1 h. After centrifugation at 8,700*g* for 7 min, the supernatant was used for RNA extraction. Package liquid was collected from each of three thawed shellfish packages, and 40 ml was centrifuged at 6,000*g* for 20 min, and then the supernatant was layered onto 2 ml of 30% sucrose solution and ultracentrifuged in a P40ST rotor at 40,000 rpm for 2 h at 4°C (Hitachi Koki, Tokyo, Japan, Himac CP 80α). The pellet was resuspended in 560 μl of distilled water and used for RNA extraction. Nucleic acids were extracted with a QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) and then eluted in 60 μl elution buffer according to the manufacturer's protocol. The RNA solution was treated with 1 U of RNase-free DNase I (Takara, Shiga, Japan) for 10 min at 37°C, after which the enzyme was inactivated by treatment at 75°C for 5 min. RT was performed with 10 μl of RNA solution with or without DNase I treatment and 30 μl of RT mixture containing 2 mM dNTPs mixture, 20 mM dithiothreitol, 0.3 μg of random nonamer primer (Takara), 1 μg of oligo $pd(T)_{12-18}$ (Invitrogen, Tokyo, Japan), 40 U of RNase inhibitor (Takara), 400 U of M-MLV reverse transcriptase (Invitrogen) and 8 μl of 5 × first-strand buffer (Invitrogen).

Real-Time PCR and Nested PCR

For SaV, MGB TaqMan®-based real-time PCR, which detects all human genogroups, and nested PCR for SaV were carried out as previously described [Oka et al., 2006; Okada et al., 2006]. Primers F13, F14, R13, and R14 were used for the first PCR, and then primers F22 and R2 were used for the nested PCR. In real-time PCR, samples were determined to be positive for SaV when the copy number in 5 μl of cDNA template was greater than 25 using standard plasmid DNA [Oka et al., 2006]. For NoV, genogroup-specific TaqMan-based real-time PCR and genogroup-specific semi-nested PCR were carried out as previously described [Kageyama et al., 2003; Hansman et al., 2008]. For genogroup I (GI), COG1F and G1SKR primers [Kojima et al., 2002; Kageyama et al., 2003] were used for the first PCR, and then G1SKF and G1SKR primers [Kojima et al., 2002] were used for the nested PCR. For NoV genogroup II (GII), COG2F and G2SKR primers [Kojima et al., 2002; Kageyama et al., 2003] were used for the first PCR, and then G2SKF and G2SKR primers [Kojima et al., 2002] were used for the nested PCR. In real-time PCR, samples were determined to be positive for NoV when the copy number in 5 μl of cDNA template was greater than 10 using a standard plasmid DNA [Nishida et al., 2003].

The cDNA prepared from DNaseI-treated RNA was used for the detection of NoV in human feces, the digestive diverticulum of stripped shellfish and the package liquid and for detection of SaV in feces. To detect the SaV genome from the digestive diverticulum of stripped shellfish or the package liquid, cDNA prepared from RNA not treated with DNase I was used as a template, and the number of cycles for nested PCR was increased from 35 to 50.

Nucleotide Sequencing and Phylogenetic Analysis

The PCR products amplified with the F22 and R2 primer set for SaV, the COG1F and G1SKR primer set for NoV GI from stools, the G1SKF and G1SKR primer set for NoV GI from clams, the COG2F and G2SKR

primer set for NoV GII from stools, and the G2SKF and G2SKR primer set for NoV GII from clams were excised from the gel and purified by a QIAquick gel extraction kit (Qiagen) or a QIAquick PCR purification kit (Qiagen) and then cloned into a pCR2.1 vector (Invitrogen). The insert was amplified with M13-RV primer (5′-CAGGAAACAGCTATGAC-3′) and M13-20 primer (5′-GTAAAACGACGGCCAG-3′), and the PCR products were separated by 2% agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Qiagen), and at least four clones from each specimen were sequenced directly with M13-20 and -RV primers by a 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan) with a Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems). Nucleotide sequences were assembled with SEQUENCHER version 4.7 (Gene Codes Corporation, Ann Arbor, MI) and aligned with Clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-j.html). A representative sequence(s) from each specimen was used for a homology search (BLAST) and phylogenetic analysis. A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method. The distance of the nucleotide substitutions per site was calculated by Kimura's two-parameter method and illustrated using NJPlot software (http://pbil.univ-lyon1.fr/software/njplot.html).

Nucleotide Sequence Accession Numbers

The SaV and NoV nucleotide sequences determined in this study have been deposited in DDBJ under the accession numbers AB522390-AB522424.

RESULTS

Description of Outbreak

An outbreak of gastroenteritis occurred among restaurant guests (groups A, B, and C) on June 6 and guests of a party (group D) who ate catered food on June 7, 2008. Thirty-eight (76%) of the 50 guests had clinical symptoms associated with gastroenteritis. The attack rates were 75% (21 of 28) in group A, 87.5% (7 of 8) in group B, 67% (2 of 3) in group C, and 72.7% (8 of 11) in group D. None of the 10 food handlers who worked at the restaurant had gastroenteritis symptoms.

Thirty-four stool specimens were collected from the 60 people (50 guests and 10 food handlers), including 23 specimens from symptomatic guests (13 in group A, 5 in group B, and 5 in group D), one specimen from an asymptomatic guest in group A, and 10 specimens from asymptomatic food handlers (Table I). Stool specimens were not collected from group C. The symptoms of 23 symptomatic guests available for pathogen screening were diarrhea (78.3%), vomiting (60.9%), abdominal pain (60.9%), and fever (higher than 37.5°C) (26.1%) (Table I). The incubation periods were from 5 to 60.5 h (median, 36.0 h) (Table I).

Through interviews with the guests by food inspectors, salmon and clam au gratin were identified as the common foods in groups A, B, and C; the latter was suspected to be a source of this outbreak because the members of groups A, B, and C claimed that the onion in the clam au gratin was undercooked. While the members of group D did not eat the clam au gratin, the catered food for group D was prepared on the same cooking counter on June 6 and stored in a refrigerator. In the restaurant, imported frozen stripped shellfish was used to prepare the clam au gratin (5 stripped shellfish per one clam au gratin). Based on this information, it was suspected that the gastroenteritis outbreak in groups A, B, and C occurred from the consumption of undercooked shellfish and that the outbreak in group D occurred from the consumption of some food(s) which were contaminated during preparation in the same kitchen. The fecal specimens were screened initially for enteropathogenic bacterial pathogens, *Salmonella* spp., *Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Campylobacter* spp., *Vibrio cholerae*, and *Shigella* spp.; however, all specimens were negative (data not shown).

Virus Detection in Fecal Specimens From Patients and Food Handlers

Of 24 fecal specimens collected from the guests, 12 were positive for SaV (including a specimen from an asymptomatic guest, D1729) by nested RT-PCR, and 11 were positive for NoV by semi-nested RT-PCR, of which 9 were GI and 4 were GII (2 positive for both GI and GII). Five patients, D1739 in group A, D1714 in group B, and D1734, D1741, and D1743 in group D, were positive for both SaV and NoV (Table II). Thirteen guests (D1711, D1712, D1729, D1730, D1731, D1732, D1736, D1737, and D1738 from group A, D1715, D1716, and D1718 from group B, and D1742 from group D) were positive for just one virus, NoV or SaV (Table II). The numbers of viral cDNA copies per gram of fecal specimen determined by real-time RT-PCR ranged from 3.46×10^5 to 2.09×10^{10} for SaV, 4.4×10^6 to 2.0×10^7 for NoV GI and 9.6×10^6 to 3.9×10^9 for NoV GII (Table II). One of the 10 asymptomatic food handlers (D1725) was positive for NoV GII by both semi-nested PCR and real-time PCR (1.4×10^6 copies per gram of fecal specimen) (Table II).

Virus Detection in Stripped Shellfish and the Package Liquid

SaV and NoV were detected by PCR from the shellfish and the package liquids collected from three individual stripped shellfish packages (Package-1, -2, and -3) (Table II). The shellfish from Package 1 were positive for both SaV and NoV GII by RT-nested PCR and/or real-time RT-PCR. The liquid from Package 1 was also positive for both SaV and NoV GI. The liquid from Package 2 was positive for SaV by nested RT-PCR and the digestive diverticulum from Package 3 was positive for NoV GI by semi-nested RT-PCR (Table II). SaV in stripped shellfish or the package liquid was positive only

TABLE I. Description of Guests and Food Handlers Associated With the Outbreak

Group	Sample ID	Onset of illness	Incubation period (hr)	Specimen collected date	Sex	Age	Diarrhea	Vomiting nausea	Abdominal pain	Fever[a]
A	D1710	June 7, 2008	11	June 10, 2008	M	54	−	+	−	−
	D1711	June 8, 2008	37	June 10, 2008	F	47	+	+	+	−
	D1712	June 7, 2008	23	June 10, 2008	M	27	+	+	+	+
	D1713	June 8, 2008	49	June 10, 2008	M	57	+	−	+	−
	D1729	—	—	June 12, 2008	F	31	−	−	−	−
	D1730	June 7, 2008	5	June 11, 2008	F	42	−	−	+	−
	D1731	June 8, 2008	47	June 12, 2008	F	27	+	+	+	+
	D1732	June 8, 2008	38	June 12, 2008	F	21	+	−	+	−
	D1735	June 7, 2008	13	June 13, 2008	F	37	+	+	−	−
	D1736	June 8, 2008	34.5	June 13, 2008	F	40	+	+	−	−
	D1737	June 8, 2008	37	June 13, 2008	M	25	+	+	+	+
	D1738	June 7, 2008	14	June 13, 2008	F	29	+	−	+	−
	D1739	June 8, 2008	43.5	June 13, 2008	F	27	+	+	−	−
	D1740	June 8, 2008	36	June 13, 2008	F	22	+	−	−	−
B	D1714	June 9, 2008	60.5	June 10, 2008	M	58	+	−	−	+
	D1715	June 8, 2008	49.5	June 10, 2008	M	56	+	−	+	−
	D1716	June 7, 2008	13.5	June 10, 2008	M	—	+	+	+	−
	D1717	June 7, 2008	13.5	June 10, 2008	M	59	+	−	+	+
	D1718	June 8, 2008	43.5	June 10, 2008	M	59	−	−	+	−
D	D1733	June 8, 2008	34	June 12, 2008	M	—	+	+	−	−
	D1734	June 9, 2008	40.5	June 12, 2008	F	—	−	+	−	−
	D1741	June 8, 2008	33	June 13, 2008	F	—	+	+	+	−
	D1742	June 9, 2008	36	June 13, 2008	M	—	+	+	+	−
	D1743	June 9, 2008	43	June 13, 2008	F	—	−	+	−	+
Food handler	D1719	—	—	June 10, 2008	M	51	−	−	−	−
	D1720	—	—	June 10, 2008	M	49	−	−	−	−
	D1721	—	—	June 10, 2008	M	30	−	−	−	−
	D1722	—	—	June 10, 2008	M	28	−	−	−	−
	D1723	—	—	June 10, 2008	M	38	−	−	−	−
	D1724	—	—	June 10, 2008	M	34	−	−	−	−
	D1725	—	—	June 10, 2008	M	57	−	−	−	−
	D1726	—	—	June 10, 2008	M	26	−	−	−	−
	D1727	—	—	June 11, 2008	M	59	−	−	−	−
	D1728	—	—	June 11, 2008	M	52	−	−	−	−

[a]Higher than 37.5°.

when cDNA prepared from nucleic acid without DNase I treatment was used as a template for RT-PCR and/or RT-real-time PCR (data not shown).

Phylogenetic and Nucleotide Sequence Analyses

Nucleotide sequences of 8 GI SaV, 9 GII SaV, 12 GI NoV, and 6 GII NoV strains were divided into three, two, two, and five different branches, respectively, when their phylogenetic trees were constructed by NJplot analysis based on the partial capsid nucleotide sequences (Figs. 1 and 2). Among these branches, there were three clusters for SaVs (2 GI clusters and 1 GII cluster: cluster A including D1729-A, D1743-D-a, and Package 1-Liquid, cluster B including D1736-A, D1734-D, and Asari1-DT-a, and cluster C [the GII cluster] including D1737-A, D1739-A, D1743-D-b, Asari1-DT-b, and Package 2-Liquid), one cluster for NoV GI designated as cluster A (D1712-A, D1730A, D1731-A, D1739-A, D1715-B, D1716-B, D1734-D, D1741-D-b, D1742-D, Package 1-Liquid, and Asari3-DT), and one cluster for NoV GII designated as cluster B (D1714-B and Asari1-DT). The nucleotide sequence identities of the PCR products for SaV (405 bp) between patients and digestive diverticulum and/or package liquid were 100% in 2 GI clusters (designated as clusters A and B) and 99.3–99.8% (1–3 nucleotide difference) in one GII cluster (designated as cluster C) for SaV. Similarly, they were 98.6–99.7% (1–4 nucleotide difference) and 99.3% (2 nucleotide difference) in the NoV GI cluster (designated as cluster A) and the NoV GII cluster (designated as cluster B), respectively, when 295 or 282 bp were analyzed, respectively. These results showed very similar or identical nucleotide sequences among the fecal specimens from the symptomatic or asymptomatic guests and digestive diverticulum and/or the liquid collected from the frozen stripped shellfish packages.

DISCUSSION

It has been suspected that SaV can be transmitted by shellfish and cause subsequently outbreaks of gastroenteritis because SaVs were detected from shellfish which were purchased from markets [Hansman et al., 2007a] and also detected in stool specimens that were collected from gastroenteritis patients associated with the consumption of shellfish [Nakagawa-Okamoto

TABLE II. Detection of Norovirus and Sapovirus in Fecal Specimens From Guests, Food Handlers, and From Shellfish Specimens

		Sapovirus				Norovirus GI				Norovirus GII			
Group	Sample ID	Viral load[a]	Nested RT-PCR	Strain name	Accession no.	Viral load[a]	Semi-nested RT-PCR	Strain name	Accession no.	Viral load[a]	Semi-nested RT-PCR	Strain name	Accession no.
A	D1710	—	–			—	–			—	–		
	D1711	1.90×10^8	+	D1711-A/Jun2008/JPN_2	AB522391	—	–			—	–		
	D1712	—	–			1.2×10^7	+	D1712-A-a/Jun2008/JPN_1	AB522407	9.6×10^6	+	D1712-A-b/Jun2008/JPN_13	AB522419
	D1713	—	–			—	–			—	–		
	D1729	2.09×10^{10}	+	D1729-A/Jun2008/JPN_4	AB522393	—	–			—	–		
	D1730	—	–			2.5×10^6	+	D1730-A/Jun2008/JPN_5	AB522411	—	–		
	D1731	—	–			9.6×10^6	+	D1731-A/Jun2008/JPN_6	AB522412	—	–		
	D1732	1.23×10^7	+	D1732-A/Jun2008/JPN_5	AB522394	—	–			—	–		
	D1735	—	–			—	–			—	–		
	D1736	6.70×10^9	+	D1736-A/Jun2008/JPN_7	AB522396	—	–			—	–		
	D1737	1.21×10^{10}	+	D1737-A/Jun2008/JPN_8	AB522397	—	–			—	–		
	D1738	7.42×10^8	+	D1738-A/Jun2008/JPN_9	AB522398	—	–			—	–		
	D1739	2.44×10^8	+	D1739-A/Jun2008/JPN_10	AB522399	1.2×10^7	+	D1739-A/Jun2008/JPN_8	AB522414	—	–		
	D1740	—	–			—	–			—	–		
B	D1714	—	+	D1714-B/Jun2008/JPN_1	AB522390	—	–			1.3×10^8	+	D1714-B/Jun2008/JPN_14	AB522420
	D1715	—	–			2.0×10^7	+	D1715-B-a/Jun2008/JPN_2 D1715-B-b/Jun2008/JPN_3	AB522408 AB522409	—	–		
	D1716	—	–			2.0×10^7	+	D1716-B/Jun2008/JPN_4	AB522410	—	–		
	D1717	—	–			—	–			—	–		
	D1718	3.50×10^8	+	D1718-B/Jun2008/JPN_3	AB522392	—	–			—	–		
D	D1733	—	–			—	–			—	–		
	D1734	3.24×10^8	+	D1734-D/Jun2008/JPN_6	AB522395	2.0×10^7	+	D1734-D/Jun2008/JPN_7	AB522413	—	–		
	D1741	3.87×10^7	+	D1741-D/Jun2008/JPN_11	AB522400	—	+	D1741-D-a/Jun2008/JPN_9	AB522415	3.9×10^9		D1741-D-b/Jun2008/JPN_16	AB522422
	D1742	—	–			4.4×10^6	+	D1742-D/Jun2008/JPN_10	AB522416	—	–		
	D1743	3.46×10^5	+	D1743-D-a/Jun2008/JPN_12 D1743-D-b/Jun2008/JPN_13	AB522401 AB522402	—	–			1.5×10^9	+	D1743-D/Jun2008/JPN_17	AB522423
Food handler	D1719	—	–			—	–			—	–		
	D1720	—	–			—	–			—	–		
	D1721	—	–			—	–			—	–		
	D1722	—	–			—	–			—	–		
	D1723	—	–			—	–			—	–		
	D1724	—	–			—	–			—	–		

(*Continued*)

TABLE II. (*Continued*)

Group	Sample ID	Sapovirus Viral load[a]	Sapovirus Nested RT-PCR	Sapovirus Strain name	Sapovirus Accession no.	Norovirus GI Viral load[a]	Norovirus GI Semi-nested RT-PCR	Norovirus GI Strain name	Norovirus GI Accession no.	Norovirus GII Viral load[a]	Norovirus GII Semi-nested RT-PCR	Norovirus GII Strain name	Norovirus GII Accession no.
	D1725	—	–			—	–			1.4×10^6	+	D1725/Jun2008/JPN_15	AB522421
	D1726	—	–			—	–			—	–		
	D1727	—	–			—	–			—	–		
	D1728	—	–			—	–			—	–		
Ruditapes philippinarum (Asari)													
Package 1	DT 1[b]	+	+	Asari1-DT-a/ Jun2008/JPN_14	AB522403	—	–			+	+	Asari1-DT/ Jun2008/JPN_18	AB522424
				Asari1-DT-b/ Jun2008/JPN_15	AB522404								
	Liquid 1	—	+	Asari1-Liquid/ Jun2008/JPN_16	AB522405	—	+	Asari1-Liquid/ Jun2008/JPN_12	AB522418	—	–		
Package 2	DT 2[b]	—	–			—	–			—	–		
	Liquid 2	—	+	Asari2-Liquid/ Jun2008/JPN_17	AB522406	—	–			—	–		
Package 3	DT 3[b]	—	–			—	+	Asari3-DT/ Jun2008/JPN_11	AB522417	—	–		
	Liquid 3	—	–			—	–			—	–		

[a]Number represents copies/g feces.
[b]DT indicates digestive diverticulum.

Fig. 1. Phylogenetic tree of SaV based on partial capsid nucleotide sequences. Seventeen SaVs detected in this study are shown in bold face on the tree. Clusters A and B in genogroup I and cluster C in genogroup II in this study are boxed. The numbers on each branch indicate the bootstrap values for the cluster, where values of 950 or higher were considered sufficiently significant for grouping. The scale indicates the nucleotide substitutions per site.

et al., 2009]. In this study, identical or very similar SaV nucleotide sequences (~99.3% nucleotide identity) were demonstrated on the basis of partial capsid nucleotide sequences in patients and shellfish from the suspected batch, similar to the case of NoV. The results strongly suggested food-to-human transmission for SaV in addition to NoV by shellfish cooked inadequately. However, further investigation is needed for determining the relation between SaVs found in shellfish and patients using variable region sequences such as the NoV P2 domain to show the identity of the genome.

SaV and NoV have been detected from feces of symptomatic patients with oyster-associated gastroenteritis; however, the details of clinical symptoms were not available [Nakagawa-Okamoto et al., 2009]. Therefore, it was interesting to determine whether multiple virus infections cause severe clinical symptoms. Clinical symptoms and the incubation periods of patients infected with multiple species of viruses were not different from those of patients infected with a single species of virus, although the number of patients compared was limited (Tables I and II). In addition, an

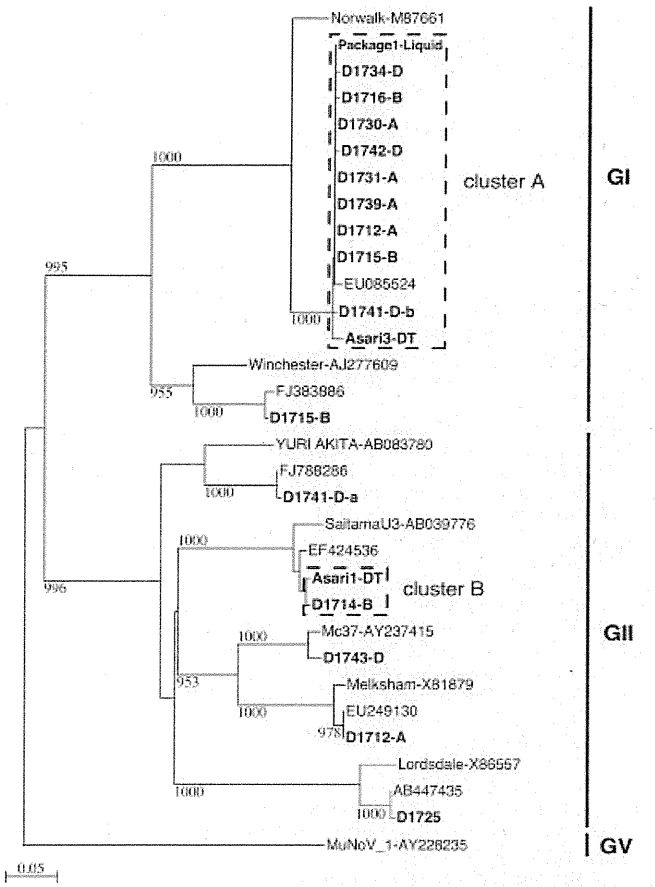

Fig. 2. Phylogenetic tree of NoV based on partial capsid nucleotide sequences. Eighteen NoVs detected in this study are shown in bold face on the tree. Cluster A in genogroup I and cluster B in genogroup II in this study are boxed. The numbers on each branch indicate the bootstrap values for cluster, where values of 950 or higher were considered sufficiently significant for grouping. The scale indicates the nucleotide substitutions per site.

asymptomatic guest (D1729) had the highest SaV RNA level among those providing fecal specimens (Tables I and II). These results suggest that there is no direct relation between combined infection of NoV and SaV and particular or severe gastroenteritis symptoms, although further investigation is necessary.

The presence of genetically diverse NoV and SaV strains in shellfish is well known. Indeed, NoV and SaV with various nucleotide sequences were detected in shellfish samples examined in this study (Figs. 1 and 2). The viral load of NoV and SaV in the stripped shellfish or the package liquid seemed to be low because it was mostly below the detection limit by real-time RT-PCR (Table I). Nevertheless, it is likely that SaV and NoV concentrated in shellfish or leaked into the package liquid are capable of transmission to humans and causing gastroenteritis.

Based on a BLAST search, Package 1-Liquid (cluster A), Asari1-DT-a (cluster B), and Package 2-Liquid (cluster C) for SaV (Fig. 1) were close to Yokote1 (AB253740) [Hansman et al., 2007b] detected in a 5-year-old female in 2006 in Japan, oyster/Miyagi/1/2009 (AB510019) detected from an oyster (*Crassostrea gigas*) in 2009 in Japan (AB510019), and Kumamoto6/2003 (AB429084) detected in a 1-year-old female in 2003 in Japan [Harada et al., 2009] with 99%, 98%, and 99% nucleotide identities, respectively, when approximately 350–400-nt partial capsid nucleotide sequences were compared (data not shown). Package 1-Liquid, belonging to cluster A for NoV GI (Fig. 2), was close to EU085524 detected from mussels (*Mytilus edulis*) in 2004 in Sweden [Nenonen et al., 2008], and Asari1-DT, belonging the cluster B for NoV GII (Fig. 2), was close to EF424536 detected from clams (*Corbicula japonica*) in 2006 in Japan [Hansman et al., 2008] when approximately 280–290-nt partial capsid nucleotide sequences were compared. These results suggest that genetically similar SaVs and NoVs are distributed in various areas of Japan and abroad.

While NoV GII sequence was detected from an asymptomatic food handler (D1725), the same sequence was not detected in any of the guests (Fig. 2). Therefore, the nucleotide sequence analysis clearly ruled out the possibility that this food handler was the source of infection in this case.

In conclusion, we detected SaV and NoV from patients with gastroenteritis and the suspected food (shellfish). To control SaV and NoV infection by intake of shellfish, their stability against heat and the criteria for viruses in shellfish and harvest areas should be clarified.

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ORIGINAL ARTICLE

Novel monoclonal antibodies broadly reactive to human recombinant sapovirus-like particles

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ABSTRACT

Sapovirus (SaV), a member of the family *Caliciviridae*, is an important cause of acute epidemic gastroenteritis in humans. Human SaV is genetically and antigenically diverse and can be classified into four genogroups (GI, GII, GIV, and GV) and 16 genotypes (7 GI [GI.1–7], 7 GII, [GII.1–7], 1 GIV and 1 GV), based on capsid sequence similarities. Monoclonal antibodies (MAbs) are powerful tools for examining viruses and proteins. PAI myeloma cells were fused with spleen cells from mice immunized with a single type of recombinant human SaV virus-like particles (VLPs) (GI.1, GI.5, GI.6, GII.3, GIV, or GV). Sixty-five hybrid clones producing MAbs were obtained. Twenty-four MAbs were characterized by ELISA, according to their cross-reactivity to each VLP (GI.1, GI.5, GI.6, GII.2, GII.3, GII.4, GII.7, GIV, and GV). The MAbs were classified by this method into: (i) MAbs broadly cross-reactive to all GI, GII, GIV and GV strains; (ii) those reactive in a genogroup-specific; and (iii) those reactive in a genotype-specific manner. Further analysis of three broadly cross-reactive MAbs with a competitive ELISA demonstrated that at least two different common epitopes are located on the capsid protein of human SaVs in the four genogroups. The MAbs generated and characterized in this study will be useful tools for further study of the antigenic and structural topography of the human SaV virion and for developing new diagnostic assays for human SaV.

Key words cross-reactivity, monoclonal antibody, sapovirus.

Sapovirus, a member of the family *Caliciviridae*, causes gastroenteritis in humans and is a significant public health problem (1–5). SaV was originally identified by EM of fecal specimens obtained during a gastroenteritis outbreak (6, 7).

The SaV capsid is composed of 90 dimers of capsid protein (VP1) (8). SaV has a ~7.5 kb genome of single-stranded positive-sense RNA that is predicted to encode two or three ORFs. The functions of proteins encoded by ORF2 and ORF3 are unknown. However, ORF1 encodes nonstructural proteins and VP1 (9, 10). VP1 is likely produced by cleavage of the ORF1 polyprotein by viral protease or by translation from subgenomic RNA (3′-coterminal with the virus genome), or both (11, 12). A tripeptide, MEG, conserved among human SaV strains, is probably the putative VP1 start on the subgenomic RNA.

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List of Abbreviations: CBB, Coomassie brilliant blue; DAB, diaminobenzidine; EM, electron microscopy; G, genogroup; HRPO, horseradish peroxidase; MAb, monoclonal antibody; NoV, norovirus; OD, optical density; OPD, ortho-phenylenediamine; ORF, open reading frame; P domain, protrusion domain; TPBS, Tween 20 phosphate-buffered saline; S domain, shell domain; SaV, sapovirus; *Sf9*, *Spodoptera frugiperda*; VLP, virus-like particles; VP1, capsid protein; WB, western blotting.

VP1 expressed from the putative subgenomic RNA or putative VP1-encoding construct in insect or mammalian cells self-assembles into virus-like particles that are morphologically similar to native SaV (12, 13–20). SaV VP1 has an apparent molecular mass of 60 kDa (11, 12, 21). Based on their complete VP1 sequences, SaVs are classified into at least five genogroups: GI, GII, GIII, GIV and GV. GI, GII, GIV and GV infect humans, and GIII infects porcine species (9). Human SaVs can be further separated into 16 genetic clusters (seven GI [GI.1–7], seven GII, [GII.1–7], one GIV and one GV) (22).

Because there is no cell-culture system or small-animal model for human SaV, SaV VLPs have been used as models of SaV virion for immunogenic, antigenic and structural studies. The capsid proteins of human SaVs have high antigenic diversity (16, 17, 20, 21, 23). However, little information is available about whether specific regions of the VP1 are important for antigenic specificity, and whether type-specific and/or cross-reactive epitopes are present in SaVs.

Monoclonal antibodies are powerful tools for the study of viruses and proteins. A panel of MAbs against SaV VLPs would be valuable for antigenic and structural analysis as well as useful for developing new diagnostic assays for human SaV. In this study, we established such a panel of MAbs broadly cross-reactive to all human SaV genogroups, GI, GII, GIV and GV, as well as MAbs specific to either genogroups or genotypes.

MATERIALS AND METHODS

Generation of recombinant baculoviruses

DNA fragments corresponding to the putative subgenomic RNA region of the genome (approximately 2.3 kb in length) of GI.6 Nichinan (GenBank accession number AB455803 [24]), GII.3 20082029 (AB630068 [22]), GII.3 D1711 (AB522391 [25]), GII.3 Kushiro5 (AB455793 [26]), GII.3 Nayoro4 (AB455794 [26]), GII.4 Kumamoto6 (AB429084 [1, 22]), GII.7 20072248 (AB630067 [22]), and GIV Yakumo8 (AB455795 [26]) were amplified by PCR with KOD-Plus-DNA polymerase (Toyobo) as previously described (27). A forward primer (5′-CAGATCTGCA GCGGCCGCATGGAGGN$_{8-10}$ [N indicates strain specific sequence]-3′) included a NotI site (underlined), and a common reverse primer (5′-GTCCCAGGAAAGGATCC TTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′) included a BamHI site (underlined). The amplified fragments were cloned into NotI- and BamHI-digested baculovirus transfer vector pVL1392 (Orbigen, San Diego, CA, USA) with an In-Fusion Advantage PCR Cloning Kit (Takara, Shiga, Japan), according to the manufacturer's protocol. *Escherichia coli* HST08 premium competent cells (Takara) were used to transform and propagate the transfer plasmid. Sequencing analysis confirmed the consensus sequence of each strain. An insect cell line derived from *Sf9* (Riken Cell Bank, Tsukuba, Japan) was co-transfected with a linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Baculo-Gold, BD Bioscience, Franklin Lakes, NJ, USA) and the transfer vectors carrying the human SaV putative subgenomic RNA region by the lipofectin-mediated method as specified by the manufacturer (Gibco BRL, Gaithersburg, MD, USA).

Expression and purification of human sapovirus virus-like particles

For larger scale expression of the SaV capsid proteins, BTl-Tn-5B1–4 (Tn5), an insect cell line derived from *Trichoplusia ni* (Invitrogen, San Diego, CA, USA), was infected with recombinant baculoviruses at a multiplicity of infection of 10 and incubated for 7 days at 26°C, as previously described (28). Eight novel VLPs derived from GI.6 Nichinan, GII.3 20082029, GII.3 D1711, GII.3 Kushiro5, GII.3 Nayoro4, GII.4 Kumamoto6, GII.7 20072248 and GIV Yakumo8 were purified as follows. Intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 *g* for 60 min. The supernatant was then centrifuged at 174,899 *g* for 3 hr in a Beckman SW32-Ti rotor rotor (Beckman Coulter, Fullerton, CA, USA). The resulting pellet was resuspended in EX-CELL 405 Serum Free medium (SAFC Biosciences, Lenexa, KS, USA) at 4°C overnight and the debris removed by centrifugation at 10,000 *g* for 30 min at 4°C. The supernatant was then centrifuged at 154,000 *g* for 2 hr in a Beckman TLA55. The resulting pellet was resuspended in EX-CELL 405 Serum Free medium (SAFC Biosciences) at 4°C overnight, and the debris removed by centrifugation at 10,000 *g* for 5 min at 4°C. After mixing with 2.1 g of CsCl in MilliQ water, the sample was centrifuged at 148,862 *g* for 24 hr at 10°C in a Beckman SW55-Ti rotor. After fractionation (20 × 250 μL each), each aliquot was diluted with EX-CELL 405 medium, and centrifuged at 154,000 *g* for 2 hr at 4°C in a Beckman Coulter TLA55 rotor. The resulting pellet was resuspended in EX-CELL 405 medium. Seven VLPs derived from GI.1 Mc114 (AY237422 [27]), GI.5 Yokote1 (AB253740 [17]), GII.2 Mc10 (AY237420 [10]), GII.3 C12 (AY603425 [10]), GII.3 Syd53 (DQ104360 [29]), GIV Syd3 (DQ104357 [29]) and GV NK24 (AY646856 [30]) were expressed and purified as previously described (14, 16, 17, 20).

Preparation of monoclonal antibodies

The PAI myeloma cell line (kindly provided by M. Kotani, Tokyo Metropolitan Institute of Medical Science,

Tokyo, Japan) was cultured in Dulbecco's modified Eagle's medium with 15% FCS. The MAbs were prepared essentially as previously described (31, 32), with minor modifications. Briefly, BALB/c mice were primed intraperitoneally with 1–10 μg of purified VLPs (GI.1 Mc114, GI.5 Yokote1, GI.6 Nichinan, GII.3 Syd53, GIV Syd3, or GV NK24 VLPs) per mouse, in the presence of adjuvant. The mice received booster inoculations four times at 1 week intervals, the final injection of antigen being administered i.v. Three to four days after the final injection, the animals were killed and cells from their spleens fused with the myeloma cells. The culture medium of the hybridomas that resulted from successful fusions was screened for reactivity by ELISA. ELISA plates were coated with VLPs as described below. Positive hybridomas were cloned by limiting dilution and antibody-producing clones were grown and stored in liquid nitrogen until used for further tests. Finally, ascites fluid was prepared by injecting the hybridomas into pristane-primed mice and used to provide the MAbs for this study. The isotype and subclass of each MAb were determined by ELISA with anti-mouse subtype MAbs (Cappel Laboratories, West Chester, PA, USA) or an IC kit (IsoQuick, Sigma, Saint Louis, MO, USA).

All animal procedures conformed to the Animal Handling and Ethical Regulations of the University of Hyogo and the provisions of the Declaration of Helsinki. This research project was approved by the Ethics Committee of the University of Hyogo.

Enzyme-linked immunosorbent assay

An indirect ELISA, with slight modifications, was used to screen and characterize the MAbs (31). Briefly, 96-well microplates (Nunc-immune plate, Nunc, Roskilde, Denmark) were coated with 100 ng of VLPs/well in 50 μL of PBS (pH 7.2) overnight at 4°C. The plates were washed with TPBS and blocked with 5% skim milk in PBS for 1 hr at 37°C. The MAbs (ascites, appropriate dilution, 50–100 μL) were added and incubated for 1 hr at 37°C. After washing with TPBS, 50 μL of a 1:2000 dilution of HRPO–conjugated goat anti-mouse immunoglobulin G (IgG), IgM, or IgA (Cappel Laboratories) was added to each well and incubated for 1 hr at 37°C. After washing, 50 μL of OPD-H_2O_2 (0.5 mg of ortho-phenylenediamine/mL, 0.002% H_2O_2, 0.1 M citrate-phosphate buffer, pH 5.5) was added, incubated for 10–20 min, and the optical densities measured at 490 and 655 nm with a Microplate Reader (Model 550, Bio-Rad, Richmond, CA, USA).

To further characterize the epitopes recognized by these MAbs, a competitive indirect ELISA was performed as previously described (33, 34) with a slight modification. Briefly, VLP was used to coat 96-well microplates overnight at 4°C at a concentration of 50–100 ng/well in PBS (pH 7.2). In separate tubes, MAbs at a concentration of 5–500 ng/mL (depending on the VLPs used for the coating) were added to decreasing concentrations of competitor VLP (10, 1, 0.1 and 0.01 μg/mL) in PBS (pH 7.2) containing 1% skim milk, and then incubated overnight at 4°C. As a control, MAb without competitor VLP was included in each plate. The VLP-coated plates were washed and blocked with 5% skim milk for 1 hr at 37°C, 100 μL of each of the VLP-MAb reaction mixtures was added to duplicate wells, and the plates were incubated for 2 hr at 37°C. The reactivity of the antibody to the competitors was expressed as B/B_0, where B is the amount of antibody bound to the coating antigen in the presence of the competitor, and B_0 is the amount in the absence of the competitor.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The VLPs (0.5–1 μg per track) were suspended in electrophoresis sample buffer containing 1% SDS, 10% 2-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), 0.0025% phenol red, and 10% glycerol. The samples were boiled for 2 min, then subjected to 10% SDS-PAGE (catalog no. EMP-8020; 1 mm thick, 8 cm long, 7 cm wide; Iwaki, Tokyo, Japan) at a constant current of 20 mA for 1.5–2 hr. The gels were stained with 0.1% CBB (Sigma) or silver staining kit (Ez stain Silver, Atto, Tokyo Japan).

Immunoblotting

Western blotting analysis was performed as previously described (31, 35) with slight modifications. Briefly, after electrophoresis, the gel was transferred electrophoretically to a nitrocellulose membrane (0.45 μm pore size, Millipore, Bedford, MA, USA) in a semidry transfer (EPM-8460; Iwaki) at a constant current of 70 mA for 2–3 hr. The strips were prepared and incubated overnight at room temperature with the MAbs (ascites fluid) at a dilution of 1:500–1000. The blots were incubated with a 1:2000 dilution of HRPO-conjugated goat anti-mouse IgG, IgM, and IgA (Bio-Rad) for 1 hr at 37°C. The strips were soaked in a solution of DAB (0.5 mg/mL, 3–'diaminobenzidine, 0.001% H_2O_2, 50 mM Tris-HCl buffer, pH 6.0) to detect the antigen-antibody complexes on the strips.

Sequence analysis

To confirm the sequences of the panel of plasmids used in this study, nucleotide sequence analysis was performed with a Big Dye Terminator (version 3.1) Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) and an automated sequencer, the 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were

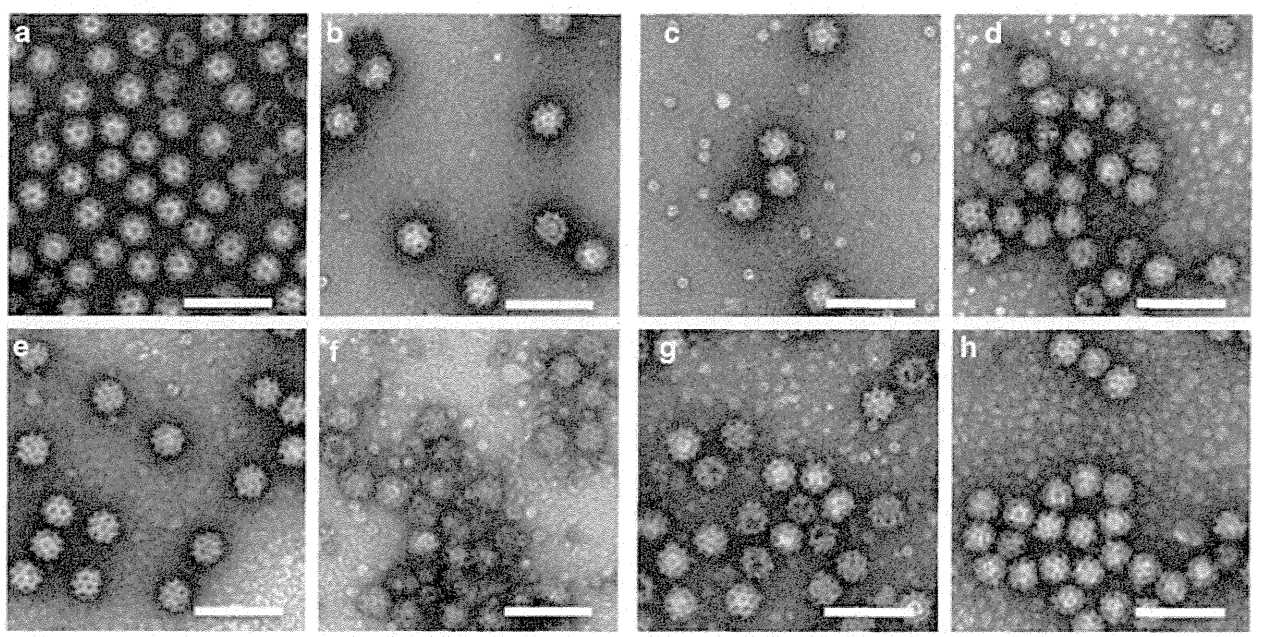

Fig. 1. Electron micrographs of novel SaV VLPs. VLPs derived from (a) SaV GI.6 Nichinan, (b) GII.3 20082029, (c) GII.3 D1711, (d) GII.3 Kushiro5, (e) GII.3 Nayoro4, (f) GII.4 Kumamoto6, (g) GII.7 20072248 and (h) GIV Yakumo8 have diameters of 41–43 nm. Purified VLPs were stained with 4% uranyl acetate (pH 4) and examined by an electron microscope (TEM-1400; JOEL, Japan) operating at 80 kV. Scale bars = 100 nm.

assembled with the program Sequencher, version 4.10.1 (Gene Codes, Ann Arbor, MI, USA). Nucleotide and amino acid sequences were analyzed with GENETYX Mac software, version 16.0.4 (Genetyx, Tokyo, Japan).

RESULTS

Expression of novel human sapovirus virus-like particles

Sapovirus capsid proteins were expressed in an insect cell, Tn5, and VLPs were purified by CsCl equilibrium density gradient centrifugation as described in Materials and Methods. EM analysis of eight purified SaV VLPs from GI.6 Nichinan, GII.3 20082029, GII.3 D1711, GII.3 Kushiro5, GII.3 Nayoro4, GII.4 Kumamoto6, GII.7 20072248 and GIV Yakumo8 showed cuplike surface depressions and almost homologous particles with diameters of approximately 41–43 nm (Fig. 1). The morphology of these recombinant particles is very similar to those we have observed in previous studies (14, 16, 19, 20).

Isolation of sapovirus monoclonal antibodies

BALB/c mice were immunized intraperitoneally with purified SaV VLPs and their spleen cells fused with PAI myeloma cells. Sixty-five hybrid clones producing MAbs were obtained from six different recombinant human SaV VLPs from GI.1 Mc114, GI.5 Yokote1, GI.6 Nichinan, GII.3 Syd53, GIV Syd3 or GV NK24. Twenty-four MAbs were obtained from mouse ascites and classified into five groups, according to their patterns of ELISA reactivity with 15 VLPs (GI.1 [$n = 1$], GI.5 [$n = 1$], GI.6 [$n = 1$], GII.2 [$n = 1$], GII.3 [$n = 6$], GII.4 [$n = 1$], GII.7 [$n = 1$], GIV [$n = 2$], and GV [$n = 1$]) (Table 1). The MAbs were grouped as follows: MAbs cross-reacting with all GI, GII, GIV and GV (group A, $n = 8$); GI-specific or type-specific MAbs (group B, $n = 7$); GII-specific or type-specific MAbs (group C, $n = 3$); GIV-specific MAbs (group D, $n = 2$); and GV-specific MAbs (group E, $n = 4$).

Monoclonal antibodies cross-reactive with heterologous genogroups and genotypes.

Eight group A MAbs showed binding to all GI, GII, GIV and GV VLPs examined in this study, although their reactivity to each VLP was different. Four MAbs (namely, 5C9, 1A1, 5C1 and 6C4) reacted consistently and strongly with VLPs from different genogroups and genotypes when the OD ratios between samples and PBS were greater than 10. On the other hand, another four MAbs (8127, 6D3, 3D2 and 4G7) reacted either strongly or moderately (OD ratio: 5–9) with GI, GIV and/or GV VLPs, and weakly (OD ratio 1–4) with GII VLPs in ELISA (Table 1). Among these eight MAbs, three (5C9, 1A1 and 8127) were further confirmed