合併症妊娠の予後

HIV感染合併妊娠
—周産期専門医—

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はじめに

我が国は先進国の中で唯一HIV感染者，AIDS患者が増加しているとされている。周産期医療におけるHIV感染妊娠の管理のポイントは，感染症専門医によるHIVそのものに対する治療と，産科・小児科・感染症科一体となった母子感染予防である。抗HIV薬の進歩によりHIV/AIDSは慢性疾患となり，管理を徹底すれば母子感染もほぼ予防できる時代になった。本稿では現在最良と考えられているHIV感染妊娠の管理について，具体的に述べてみたい。

HIV感染妊婦に対する妊娠中の治療の実際

妊産婦自身のAIDS発症予防ならびに母子感染予防を図る目的で，母体の血中HIVウイルス量をできるだけ低く抑えるように妊娠初期から管理を行う。妊産婦の感染判明時期や妊娠の時期，抗HIV療法の有無などにより，妊産婦やその配偶者(パートナー)，家族に十分な説明を行い，合意を得た上で管理方針を決定する。抗HIV薬の選択と投与時期の決定については内科感染症専門医に委ねるべきであり，その後も産科医と感染症専門医で連携をとって診療にあたることが大切である。

妊婦に対しても，成人に対する標準的な治療である抗HIV薬を3剤以上用いる多剤併用療法(HAART：highly active antiretroviral therapy)を選択することが基本である[1,2]。抗HIV薬を内服していない妊婦にHIV感染が判明した場合，胎児に対する影響を考慮して，妊娠14週まで(器官形成期の間)は抗HIV薬を内服せずに待ち，それ以降にAZTを含む抗HIV薬を開始する。抗HIV薬を内服している女性で妊娠が判明した場合は，妊娠14週以降であれば抗HIV薬を継続し，可能であればAZTを含んだ治療薬に変更する。妊娠14週以前に判明した場合は，抗HIV薬を継続するか，妊娠14週までは休薬するかをそれぞれのリスクを十分に説明のうえ決定する。休薬する場合はウイルス量のリバウンドや耐性ウイルスの出現などを考慮して3剤すべてを同時に中止する。しかし，一時的な休薬は再開後の母体治療を困難なものとする可能性が高く，可能であれば抗HIV薬の投与を注意深く継続することが望ましいとされる。

HIV感染症に対する抗HIV療法は，以前は催奇形性の点からAZT単剤投与が推奨されていたが，近年では耐性出現の問題などからHAARTが行われることが多い[1,2]。しかし，未だ対象例も少なく長期予後が確認されていないことから，十分なインフォームドコンセントが必要である。

抗HIV療法の治療効果を得ること，かつ薬剤耐性ウイルスの出現を防ぐために重要なことは定期的な服薬である。自覚症状がないHIV感染者に比較的副作用の頻度の高い抗HIV薬を内服させることが必要となるため，内服開始後はアドヒアランスの確認と副作用(嘔気・嘔吐，薬疹，肝機能障害，高血糖・高脂血症，貧血，乳酸アシドーシスなど)の有無を1～2週間ごとに経過観察する。

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当科ではHIV感染妊婦に対して2週間ごとの妊婦健診を行っており(表)，①カンジダ外陰腟炎，クラミジア頸管炎，ヘルペスなどの性感染症やヒトパピローマウイルスによる子宮頸癌の発生頻度が高いこと，②絨毛膜羊膜炎，切迫早産，子宮収縮，前期破水などの胎内感染のリスクを増加させる疾患の予防，③抗HIV薬の胎児への影響が未だ明らかでないため，胎児発育・胎児形態異常の評価，④検査のための頻回の採血や抗HIV薬の影響によりさらに貧血が助長されやすいこと，また胃腸管障害の強い抗HIV薬を服薬しているため，同じく胃・腸管への副作用を有する鉄剤の内服治療は困難なことが多いこと，などに留意している[1]。

HIV感染が母体・胎児に及ぼす影響

HIV感染が妊娠経過に及ぼす悪影響はなく，また妊娠がHIV感染の症状を悪化させることはないとされている[3]。また，奇形や器官形成を損なうような先天性HIV感染症候群は今までのところ報告されていない。

HIV感染妊婦の妊娠，分娩管理

HIVの母子感染には，①経胎盤感染，②産道感染，③母乳からの感染が考えられる。感染が起こりうる量のウイルスに児を被曝させない，つまり①母体のウイルス量を低下させること，②母体の血液，体液への接触を極力さけることが母子感染予防の基本である。

陣痛（子宮収縮）時には母体血（HIV汚染血液）が児へ移行しやすくなり，児は分娩中に産道からのHIVの曝露を受けやすいことから，陣痛発来前，かつ破水前に選択的帝王切開を施行する。米国を中心に母体血中のウイルス量が低い場合には選択的帝王切開は不要だとする考え方もある。しかし我が国においては，HIV感染妊娠数は年間30例程度と少なく，分娩の準備にかかる時間，大人数のスタッフの必要性など，夜間の対応も考慮すると選択的帝王切開が望ましいと思われる[1]。以前は35～36週での帝王切開を行っていたが，出生後の呼吸障害，沐浴による低体温，AZTシロップの経口投与困難などが児に認められたため，現在では，急な破水や陣痛発来に直に対応できるように妊娠35週より管理入院を行い，児の成熟を待って37～38週前半の正期産となるよう，原則として通常どおり腰椎麻酔下に帝王切開を行っている[4]。入院後，産科，感染症を専門とする内科，小児科と帝王切開数日前に合同カンファレンスを行い，図のような妊婦のプロフィールを確認し，陣痛発来，破水などの緊急の対応についても確認を行う。また手術室，麻酔科との連携も大切である。

切迫早産の場合，積極的に子宮収縮を抑制するが，帝王切開予定日を早めることも検討する。手術予定日前に陣痛発来あるいは破水した場合，直ちにAZT点滴静注を開始し，同時に帝王切開の準備を進める。分娩経過が急速で，帝王切開術に比べ経腟分娩のほうが早期に児娩出可能と判断した場合には経腟分娩とする。

帝王切開施行にあたっては，付着した母体血が児の体内に入らないように，子宮切開創からの出血を減らすように注意を払うこと，ガーゼ等で児の皮膚を十分に拭うこと，過剰な口腔内吸引で粘膜損傷を起こさないことなどに留意する必要がある。

抗HIV薬は手術前日夜まで内服とし，当日手術開始3時間前からAZT点滴投与(2 mg/kg/時で1時間投与，その後は1 mg/kg/時)を手術終了まで継続し，分娩後は経口摂取が可能となった時点で内服を再開する[1]。児には出生後8～12時間以内にAZTシロップ(2 mg/kgを6時間ごと)を6週間投与する[1,2]。

母乳中には多量のHIVが含まれるため断乳を行う[1,2]。ブロモクリプチン，テルグライド，カベルゴリンなど，通常止乳に使用される薬剤と抗ウイルス薬のプロテアーゼ阻害薬とを併用すると，これらの薬剤代謝に関与しているCYP3A4に対して競合的に作用するため，代謝が阻害されて血中濃度が上昇する可能性があり，消化器症状や精神・神

表　HIV 感染妊婦の管理手順

週数	妊娠初期	14～23 週	23 週～入院時	35～36 週	37 週	分娩後
妊婦健診	1 回/2 週	1 回/2～4 週	1 回/2 週	1 回/週		
産科管理上の注意点	分娩予定日の決定	切迫流・早産	切迫早産・IUGR 破水	入院管理 帝切準備 陣発・破水時の対応 合同カンファ	予定帝切 合同カンファ	授乳禁止 乳腺炎
検査項目				(術前検査)		(術後検査)
血液	血液型 血算 生化 凝固 感染症スクリーニング RPR TPHA HBV HCV ATL CMV ウイルス量 CD4	血算 生化 (乳酸) ウイルス量 CD4	血算 生化 (乳酸) ウイルス量 CD4	血算 生化 凝固 T&S ウイルス量 CD4	 ウイルス量 CD4	 血算 生化 (乳酸) ウイルス量 CD4
腟分泌物など	腟培 Candida GBS クラミジア 淋菌 子宮腟部細胞診	腟培(20 週前後)		腟培(35 週前後) GBS		
超音波 NST	経腟	経腹	経腹 少なくとも 1 回	経腹 1 回/週以上		
その他				胸部 X-P 心電図		
HIV 治療 (ACTG076, HAART)	内服薬の決定	14～16 週 HAART 開始			手術当日 AZT 静注	

合同カンファのメンバー：産科(医師，助産師)，小児科(医師，看護師)，感染症科(医師，コーディネーターナース)

患者病歴番号	
氏名	

年齢　　歳

HIV抗体陽性と判明した日　（　/　/　）

治療前CD4	/mm^3	（　/　/　）
治療前ウイルス量	copies/m/	（　/　/　）

抗ウイルス療法開始	（　/　/　）～
抗ウイルス療法内容	
日和見感染予防	

当院産科初診日	（　/　/　）：　週　日	
分娩予定日	（　/　/　）	
帝王切開直前CD4	/mm^3	（　/　/　）
帝王切開直前ウイルス量	copies/m/	（　/　/　）

内科合併症	
妊娠経過・特記事項	

推定体重	g	（　/　/　）：　週　日
経腹超音波所見		

帝王切開予定日	（　/　/　）：　週　日
分娩前日	（　/　）　まで抗ウイルス薬（　　）内服
当日分娩前	手術開始3時間前からAZT点滴静注射　（体重　　kg） 点滴用AZT2A　（400mg/40m/）＋5%Glu 160m/（2mg/m/）に調整 （　：　）～　　m/h　（2mg/m/）で1時間 （　：　）～　　m/h　（1mg/m/）で継続し手術終了まで
当日分娩後	経口摂取可能となったら抗ウイルス薬を再開する 母乳禁：乳房冷却 新生児：出産後8～12時間以内にAZTシロップ内服

＊切迫早産の場合：積極的に子宮収縮を抑制するが，帝王切開予定日を早めることも検討する
＊帝王切開予定日前に陣痛発来あるいは破水した場合：入院後直ちにAZTの点滴静注を開始し，同時に帝王切開の準備を勧める。分娩経過が急速で，帝王切開術に比べ経腟分娩のほうが早期に娩出可能と判断した場合には経腟分娩とする。
＊その他の連絡事項

図　合同カンファレンスのためのデータシート

経症状などの副作用を強く発現させる可能性があるため，原則として薬剤ではなく乳房のクーリングによる断乳を行っている[1,4]。

母子感染予防対策を行わなかった場合，約2割の児に感染が起こる。抗HIV療法を行ったが経腟分娩の場合は7%，選択的帝王切開を行ったが抗HIV療法を行わなかった場合は10%，抗HIV療法と選択的帝王切開を行った場合は2%の母子感染率とされる。現在，我が国における母子感染率は1.6%に抑えることが可能となっており，特に

HAARTがなされた症例では母子感染はない[1]。当科では，2006年5月までに32例のHIV感染妊娠例を経験しており，うち22例は当科において分娩，4例が他院に転院または帰国後分娩，5例が人工妊娠中絶，1例が子宮外妊娠となった。分娩した22例のうち19例はいずれも選択的帝王切開，3例は経腟分娩でいずれも母子感染は起こっていない。

HIV感染妊婦の妊娠，出産，育児には，産科医，助産師，感染症を専門とする内科医，小児科医の連携が不可欠である。当院では専任のコーディネーターナースが情報提供後の理解度の確認や，感染予防に関する日常生活上の注意点や情報の提供を行い，患者やその家族が今度の療養の見通しを立てられるよう支援を行っている[5]。

おわりに

HIV感染妊娠はきっちり管理されれば，母子感染はほぼ100％防止できるようになった。今後は母体にHAARTが行われていた場合，帝切時のAZT点滴投与や出生児へのAZT投与の必要性など，エビデンスが乏しいと考えられる項目に関するさらなる検討が必要である。

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JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2006, p. 3784–3786 Vol. 44, No. 10
0095-1137/06/$08.00+0 doi:10.1128/JCM.01373-06

Detection of Norovirus Antigens from Recombinant Virus-Like Particles and Stool Samples by a Commercial Norovirus Enzyme-Linked Immunosorbent Assay Kit

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Received 4 July 2006/Accepted 11 July 2006

The commercial norovirus enzyme-linked immunosorbent assay kit was evaluated for its reactivity to recombinant virus-like particles and the detection of natural viruses from stool samples of Japanese infants and children with sporadic acute gastroenteritis compared to reverse transcription-PCR. The kit had a sensitivity of 76.3% and a specificity of 94.9%. Our results clearly indicated that the kit allows the detection of the most prevalent genotype, GII/4. In order to increase the sensitivity of the kit, the reactivity with norovirus of GII/3 and GII/6 genotypes needs to be improved.

Norovirus (NoV) is one of the leading etiologic agents of nonbacterial sporadic acute gastroenteritis (AGE) in infants and children, and outbreaks of this infection may be due to contaminated water or food. At present, the reverse transcription-PCR (RT-PCR) assay is widely used to detect NoV in diarrheal stool samples. The development of immunological methods to detect NoV has been delayed due to the lack of viruses in cell culture and to diverse genotypes with distinct antigenicities. NoVs are currently divided into five genogroups, and most human NoV strains belong to two genogroups: genogroup I (GI) and genogroup II (GII). Furthermore, each genogroup contains at least 15 and 18 genotypes, respectively (13). RT-PCR is an expensive and complicated technique, and its use requires special equipment and skills. Thus, a faster and simpler method is needed. At present, three commercial enzyme-linked immunosorbent assay (ELISA) kits are available, the IDEIA NLV kit from Dako Cytomation, Ltd. (Ely, United Kingdom), the SRSV(II)-AD kit from Denka Seiken Co., Ltd. (Tokyo, Japan), and the RIDASCREEN norovirus (R-Biopharm AG, Darmstadt, Germany). According to previous evaluations of the ELISA kits, the first two kits cannot effectively replace RT-PCR for NoV detection due to their low sensitivities and/or specificities (1, 3, 19). To date, the RIDASCREEN norovirus assay has only been evaluated by one Australian group using outbreak specimens (2). No research has hitherto been conducted using recombinant virus-like particles (rVLPs) and sporadic stool samples.

Therefore, using the RIDASCREEN norovirus ELISA kit, we set out to measure the reactivity of 16 kinds of rVLPs, to detect the presence of NoV in fecal samples from infants and children with sporadic AGE in Japan, and to compare the sensitivity and specificity data with those obtained with the RT-PCR.

We previously expressed one rVLP (strain 1207, GII/4) (14). The other 15 rVLPs were prepared from NoV isolated from stool samples among infants and children with diarrhea between 1995 and 2003 in Japan. The genotypic classification of these NoV was performed based on the method described by Kageyama et al. (6). These are genotypes 1 (strain 4656), 3 (strain 3634), 4 (strain 2876), 8 (strain 3006), and 11 (strain 2258) in genogroup I and genotypes 1 (strain 3101), 2 (strain 2840), 3 (strain 3229), 5 (strain 3611), 6 (strain 3612), 7 (strain 419), 12 (strain 2087), 13 (strain 3385), 14 (strain 2468), and 15 (strain 3625) in genogroup II. The production of recombinant bacmids was performed using the baculovirus expression system with Gateway Technology (Invitrogen Japan, Tokyo) and the transfection of bacmids into insect cells, as well as the purification of rVLPs, was performed according to the method of Hansman et al. (5). We used two sense primers: attB1NVGI (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAT GAT GGC GTC TAA GG) for GI strains and attB1NVGII (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAA GAT GGC GTC GAA TGA) for GII strains. Purified rVLPs from the cultured supernatants of the inset cells were examined for particle formation by electron microscopy. Protein concentration of each rVLP was measured by BCA Coomassie protein assay (Pierce Biotechnology, Inc., Rockford, IL), and 150 μg/ml was prepared as stock solutions. The assays were started from 10 μg/ml as the highest concentration.

The rVLPs stock solutions were serially threefold diluted with the sample dilution buffer in the the RIDASCREEN norovirus ELISA kit and used to determine the minimal concentration of each rVLP for detection by ELISA according to the manufacturer's manual. All of the assays except that for

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TABLE 1. Minimal detected concentrations of rVLPs by ELISA

VLP	Mean minimal rVLP concn (μg/ml) ± SD[a]	
	Theoretical[b]	Detected[c]
GI/1	0.00106 ± 0.00042 (3)	
GI/3	0.0368 ± 0.0084 (3)	
GI/4		8.33 ± 2.89 (3)
GI/8	0.118 ± 0.084 (3)	
GI/11	0.0752 ± 0.0273 (3)	
GII/1	0.456 ± 0.191 (4)	
GII/2	0.116 ± 0.012 (3)	
GII/3		6.00 ± 3.65 (5)
GII/4	0.00298 ± 0.00100 (3)	
GII/5	4.22 ± 3.26 (4)	
GII/6		10 (1)
GII/7	10 < (3)*	
GII/12	0.468 ± 0.393 (3)	
GII/13	10 < (3)*	
GII/14		3.33 (2)
GII/15		6.67 ± 4.71 (2)

[a] The values show three significant figures. Each assay was done in triplicate, and the assay for single rVLP was repeated three to six times. The number of samples is given in parentheses.

[b] The theoretical values were calculated from the absorbance given by the serial dilution of rVLPs. The calculations were performed by cubic logit-log analysis ($R^2 > 0.949$). *, these rVLPs (GII/7 and GII/13) could not be determined for values of <10 μg/ml in the assay.

[c] The rVLPs given in this column could not be used to calculate the theoretical values because a theoretical value of <10 μg/ml (used maximum concentration) could not be calculated in even one assay. In these cases, the minimal detected concentrations are given.

GI/3 were done with kits of the same lot number. In the manual, the cutoff value is calculated as an absorbance value of negative control plus 0.15. Values that are 10% above or below the cutoff value are considered to be in the gray zone and therefore need to be examined again. In view of this, the theoretical minimal detectable concentration of each rVLP was determined as a calculated value which gave an absorbance value that was 10% above the cutoff value in each assay. Each assay was conducted in triplicate, and the experiment for each rVLP was repeated three to six times.

Five hundred and three stool samples were collected from infants and children with AGE who visited six pediatric clinics in Sapporo, Tokyo, Maizuru, and Osaka, Japan, from July 2004 to March 2005. All of the stool samples were stored at −30°C until testing. Watery stool samples were diluted 1:2 with phosphate-buffered saline (PBS), and hard stool samples were suspended to 1:5 with PBS. The suspensions were clarified by centrifugation at 10,000 × *g* for 15 min. The supernatants were diluted to 1:3 with the sample dilution buffer of the kit and used for the assay. The positives or negatives of the samples were determined as mentioned above.

Ten percent stool suspensions of 503 samples were prepared with PBS from the same aliquots for ELISA, and viral RNA was extracted by the QIA amp viral RNA mini kit (QIAGEN, Tokyo, Japan). The detection of NoV (GI and GII), astrovirus, sapovirus, rotavirus, and adenovirus was performed by two sets of multiplex PCR (21, 22). NoV-negative samples were examined by using two sets of monoplex PCRs, for NoV GI and GII. Twenty samples were further assayed by seminested PCR using a primer set, which were G2SKF and G2SKR for NoV GII (9). The genotypes of NoV were determined according to the method of Phan et al. (16).

TABLE 2. Sensitivity, specificity, and agreement of ELISA and RT-PCR[a]

ELISA	Detection (no. of samples) by RT-PCR		
	+	−	Total
+	87	20[b]	107
−	27	369	396
Total	114	389	503

[a] Sensitivity = 76.3% (87/114); specificity = 94.9% (369/389); agreement = 90.7% (456/503).

[b] These samples were positive as determined by seminested PCR.

The minimal detectable sensitivity is indicated in Table 1. The kit could detect GI/1 and GII/4 rVLPs at concentrations of <0.01 μg/ml. rVLPs of GI/3, GI/8, GI/11, GII/1, GII/2, and GII/12 were detectable within a range between 0.04 and 1 μg/ml. On the other hand, rVLPs of GI/4, GII/3, GII/5, GII/6, GII/14, and GII/15 were detected at more than 1 μg/ml. In cases where the assays for GI/4, GII/3, GII/6, GII/14, and GII/15 could not be detected at concentrations of <10 μg/ml and theoretical detectable concentrations could not be calculated, minimal concentrations given by the assay have been indicated (Table 1). GII/6 rVLP could be detected once at the highest concentration, 10 μg/ml. Two rVLPs of GII/7 and GII/13 could not be detected at a concentration of <10 μg/ml.

NoV in stool samples collected from sporadic cases in Japan was examined using both the ELISA kit and the RT-PCR, and the kit was evaluated based on the RT-PCR (Table 2). The calculated percent sensitivity, specificity, and agreement were 76.3, 94.9, and 90.7%, respectively. Twenty samples were determined to be positive by the kit but negative by the RT-PCR. These samples became positive when tested by the seminested PCR using NoV GII-specific primer pair. A total of 27 samples were positive with the RT-PCR but negative with the kit. The genotypes of 134 positive stool samples recorded by the RT-PCR were identified by using the clustering determined by Kageyama et al. (6) (Table 3). The genotypes of kit-positive, PCR-positive samples were 1 GI/1, 3 GII/3, 82 GII/4, and 1 GII/6, and the sensitivities of GI/1, GII/3, GII/4, and GII/6 were 50, 23.1, 85.4, and 33.3%, respectively. The low sensitivities of GII/3 and GII/6 were comparable to the results for the rVLPs. RT-multiplex PCR detected four other species of viruses in 503 stool samples. These were 7 group A rotavirus, 27 adenovirus, 30 sapovirus, and 1 astrovirus samples, and the stool samples containing these viruses were determined to be negative by ELISA. Furthermore, multiplex-PCR indicated that 8 of 112 NoV GII-positive samples were mixed infected

TABLE 3. Genotypes of norovirus in positive stool samples as determined by RT-PCR

Genogroup/ genotype	No. of samples	Frequency (%)	No. of samples positive by ELISA (%)
GI/1	2	1.8	1 (50.0)
GII/3	13	11.4	3 (23.1)
GII/4	96	84.2	82 (85.4)
GII/6	3	2.6	1 (33.3)
Total	114	100.0	87

with other viruses (5 sapovirus, 2 group A rotavirus, and 1 adenovirus).

Some studies showed that the strains belonging to GII/4 cluster were most predominant not only in stool samples from sporadic cases involving infants and children but also from the outbreaks (8, 10, 12, 15, 16, 19). On the other hand, it was found that various genotypes of NoV strains were detected in the outbreak cases, and there were no predominant genotypes in outbreak strains (20). Furthermore, a change in the distribution of NoV genotypes in the sporadic cases and the emergence of recombinant viruses has been reported (7, 11, 17, 18).

The ELISA kit could detect two kinds of rVLPs (GI/1 and GII/4) with a high sensitivity. Meanwhile, the GII/3 and GII/6 rVLPs formed a group that was responsive at higher concentrations. A total of 23.1% of the stool samples containing GII/3 NoV, and 33.3% of the samples with the GII/6 genotype could be effectively examined by the kit. NoV genotypes with low reactivity levels in the stool samples could be detected by the kit in cases with a sufficient viral load. On the other hand, the genotypes of 20 samples, which were ELISA positive and semi-nested PCR positive, were 7 GII/3 and 13 GII/4. It would appear that these samples have a smaller viral load than monoplex PCR-positive stools. This suggests that there are other factors, such as inhibitors, that may cause the lower sensitivity of ELISA.

The sensitivity, specificity, and agreement of the kit were superior to those of the Denka and Dako kits (1). Dimitriadis and Marshall showed in 2005 that the RIDASCREEN ELISA kit could not be recommended for the study of stool samples in Australian outbreaks (2). In that report, the sensitivity and specificity of the kit were 71 and 47%, respectively, with the same cutoff calculations as our own. The difference between their sensitivity value and our own, which was 76.3%, was not large. On the other hand, the specificity was very different. In the present study, the specificity of the kit based on RT-PCR assay was 94.9%. There were the false-positive samples in their results. The reason for the difference in the specificities is unclear. We have been unable to obtain either the Denka kit or the Dako kit and have not been able to compare the RIDASCREEN kit with these kits using the same stool samples.

In conclusion, our results indicated that the kit could be a useful tool for sporadic diarrheal samples. However, it is quite possible to contain many kinds of genotypes in diarrheal samples derived from food-borne sources, and the particular kinds of genotypes found in such cases are not always the same as the genotypes found in sporadic cases. All in all, the reactivity for GII/3 and GII/6 needs to be improved in order to facilitate the detection of etiological agents in outbreaks.

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences of Japan.

We thank T. Kaneshi, A. Yamamoto, S. Nishimura, S. Nakaya, and T. Nishimura for collecting stool samples.

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VIROLOGY
ELSEVIER
Virology xx (2006) xxx–xxx
www.elsevier.com/locate/yviro

Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain

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Received 12 June 2006; returned to author for revision 29 November 2006; accepted 1 December 2006

Abstract

A novel and unusual strain of porcine rotavirus (PoRV) CMP034 was isolated from a 7-week-old piglet during the epidemiological survey of porcine rotavirus infection in Chiang Mai province, Thailand from June 2000 to July 2001. Molecular characterization of gene VP4 by sequence analysis showed a low level of amino acid sequence identity, ranging from 56.7% to 76.6%, while comparison of VP8* portion showed 41.8% to 69.9% identity, with the 26 P genotypes recognized to date. Phylogenetic analysis of the VP4 sequence revealed that CMP034 was only distantly related to the other 26 P genotypes and was located in a separate branch. Sequence analysis of gene VP7 showed the highest level of amino acid identity (94.7%) with the PoRV G2-like reference strain 34461-4 but a lower level of identity with those of human G2 rotaviruses, ranging from 87.7% to 88.0%. Phylogenetic analysis of gene VP7 revealed two major lineages among G2 rotavirus strains based on the host origin. PoRV strain CMP034 clustered exclusively with G2-like PoRV strain 34461-4 in a novel lineage that is distinct from the major G2 human lineage. Moreover, strain CMP034 displayed a porcine-like VP6 and NSP5/6 with subgroup I specificity, while bearing an NSP4 with some genetic group B human-like characteristics. These findings provide evidence that CMP034 should be considered as a novel VP4 genotype P[27].

Keywords: Rotavirus; Porcine; P genotype; G genotype; Sequence analysis; Thailand

Introduction

Infection with group A rotavirus is the main cause of acute gastroenteritis in infants and young children worldwide, and in the young of many animal species, including neonatal piglets. Rotavirus belongs to the *Reoviridae* family and contains 11 segments of double-stranded RNA. The two outer-layer proteins VP7 and VP4 form the basis of the current dual classification system of group A rotavirus into G and P genotypes (Estes, 2001). To date, at least 15 G and 26 P genotypes have been identified globally, with various combinations of G and P genotypes (Estes, 2001; Martella et al., 2006a; McNeal et al., 2005; Rahman et al., 2005; Rao et al., 2000). Epidemiological studies have demonstrated that rotavirus genotypes G1, G2, G3, G4, and G9 combined with the P genotypes P[8] and P[4] are the types of VP7 and VP4 most frequently associated with human rotavirus infection globally (Gentsch et al., 1996, 2005).

In recent years, several epidemiological studies designed to monitor the appearance of novel or atypical rotavirus antigenic types have provided evidence for the increasing antigenic diversity of group A rotaviruses (Banyai et al., 2005; Gentsch et al., 2005; Yoshinaga et al., 2006). Usually, rotavirus strains sharing >89% of VP4 amino acid sequence identity are considered to belong to the same P genotype, while those sharing VP4 amino acid sequence identities <89% belong to different genotypes (Estes, 2001; Ciarlet et al., 1997; Gorziglia et al., 1990). At least 26 rotavirus P genotypes have been recognized, and the latest, P[26], was isolated from an Italian diarrheic piglet in an epidemiological study carried out in 2003–2004 (Martella et al., 2006a). Animal rotaviruses are regarded as a potential reservoir for genetic/antigenic diversity of human rotaviruses, and the potential of such transmission has been

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0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.12.004

Please cite this article as: Khamrin, P. et al. Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology (2006), doi:10.1016/j.virol.2006.12.004

reported in several studies (De Leener et al., 2004; Khamrin et al., 2006b; Nakagomi et al., 1990; Palombo, 2002). The study of animal rotaviruses is a key step to acquiring an in-depth understanding of the ecology and evolution of rotaviruses.

Group A rotaviruses are recognized as a common cause of enteric disease and gastroenteritis in neonatal piglets (Estes, 2001; Woode et al., 1976). Among porcine rotaviruses, two main P genotypes, P[6] and P[7], have been described that are commonly associated with G3, G4, G5, and G11 genotypes (Estes, 2001; Liprandi et al., 1991; Pongsuwanna et al., 1996; Winiarczyk et al., 2002). Moreover, several additional combinations of G and P genotypes have been isolated occasionally from or detected in pigs; i.e. G1, G2-like, G6, G8, G9, G10, and P[13], P[19], P[23] genotypes (Burke et al., 1994; Ciarlet and Liprandi, 1994; Gouvea et al., 1994a,1994b; Huang et al., 1993; Liprandi et al., 2003; Martella et al., 2001, 2005; Pongsuwanna et al., 1996; Racz et al., 2000).

Although G2 rotavirus strains are commonly found in humans, they were not identified from animal sources (Estes, 2001) until 2005, when PoRV strain 34461-4 bearing the G2-like genotype was reported in a piglet in Spain (Martella et al., 2005). Accordingly, a comprehensive genotypic characterization of circulating rotavirus strains among domestic animal populations is important to define the extent of rotavirus diversity.

Here, we report the isolation of a PoRV strain with a novel VP4 genotype, tentatively proposed as P[27]. Characterization of gene VP7 reveals the G2-like rotavirus genotype.

Results

Failure of both G and P genotyping of porcine rotavirus strain CMP034

During an epidemiological survey of porcine rotavirus from June 2000 to July 2001, a total of 175 fecal specimens were collected from diarrheic piglets from 6 different farms located in Chiang Mai province, Thailand. Of these, 39 (22.3%) specimens were shown by ELISA to be positive for group A rotavirus. Initial G and P genotyping was done by reverse transcription-polymerase chain reaction (RT-PCR) and multiplex-PCR assays using different pools of primers specific for human and animal VP7 and VP4 genes. Interestingly, the G and P genotypes of one rotavirus-positive specimen, CMP034, could not be identified by multiplex-PCR typing, despite that the VP7 and VP4 genes were readily amplified by RT-PCR. Therefore, the CMP034 strain was further characterized by nucleotide sequencing of genes VP4, VP6, VP7, NSP4, and NSP5/6.

VP4 and VP8 sequence analyses and determination of P genotype*

Initially, P genotype characterization of PoRV strain CMP034 by multiplex PCR genotyping was unsuccessful. In order to determine the P genotype, the forward primer Con3 was used in combination with reverse primer 170 (Martella et al., 2006a) for amplification of gene VP4 (2341 nucleotides, coding for 772 amino acids). The VP4 and VP8* nucleotide and deduced amino acid sequences of gene VP4 were compared with those of the established reference strains P[1] to P[26] available in the GenBank database (Table 1). It was observed that the nucleotide and deduced amino acid sequences of PoRV strain CMP034 shared very low levels of sequence identity with those of other P genotypes (57.4–71.9% nucleotide identity and 56.7–76.6% amino acid identity for VP4 gene, and 45.7–67.4% nucleotide identity and 41.8–69.9% amino acid identity for VP8*). Rotavirus strains that exhibit a VP4 amino acid identity of approximately >89% are considered to belong to the same P genotype, while those sharing <89% identity belong to different genotypes (Estes, 2001; Ciarlet et al., 1997; Gorziglia et al., 1990). Our results indicated that PoRV strain CMP034 was likely a novel P genotype and, therefore, tentatively proposed as a P[27] VP4 genotype.

The phylogenetic tree was constructed on the basis of the VP4 nucleotide sequence of CMP034 and those of 26 P genotypes (Fig. 1). Phylogenetic analysis clearly confirmed that

Table 1
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP4 and VP8* of porcine strain CMP034 with those of 26 known P rotavirus genotypes

Strain	Species	P genotype	VP4 identity (%)		VP8* identity (%)	
			Nucleotide	Amino acid	Nucleotide	Amino acid
A5	Bovine	P[1]	69.7	74.7	64.4	68.6
SA11	Simian	P[2]	70.5	74.7	67.3	66.5
CMH222	Human	P[3]	70.3	73.9	64.2	64.5
L26	Human	P[4]	67.2	68.5	62.1	60.2
UK	Bovine	P[5]	66.3	70.5	60.5	62.7
Gottfried	Porcine	P[6]	67.9	70.6	63.2	62.6
OSU	Porcine	P[7]	68.9	72.7	64.1	63.8
KU	Human	P[8]	69.1	68.4	63.3	60.6
K8	Human	P[9]	64.5	63.8	57.7	56.9
69M	Human	P[10]	70.7	76.6	67.4	69.9
B223	Bovine	P[11]	57.4	56.7	45.7	41.8
H-2	Equine	P[12]	70.6	74.8	66.7	66.5
MDR-13	Porcine	P[13]	69.1	70.4	61.9	60.0
PA169	Human	P[14]	63.3	64.7	55.9	56.6
Lp14	Ovine	P[15]	70.3	74.3	66.2	66.8
EW	Murine	P[16]	65.4	71.3	60.8	62.7
993/83	Bovine	P[17]	61.0	59.3	51.3	45.3
L338	Equine	P[18]	70.8	72.2	65.2	63.8
Mc323	Human	P[19]	68.5	71.1	61.7	59.5
EHP	Murine	P[20]	66.2	72.8	61.3	63.4
Hg18	Bovine	P[21]	69.6	71.1	65.8	61.4
160/01	Lapine	P[22]	60.3[a]	58.9[a]	60.3	58.9
A34	Porcine	P[23]	62.5[a]	53.5[a]	62.5	53.5
TUCH	Rhesus	P[24]	71.9	76.1	67.4	68.6
Dhaka6	Human	P[25]	63.0	63.1	55.5	55.6
134/04-15	Porcine	P[26]	69.6	72.6	63.7	66.2

The GenBank accession numbers of the following strains are given in parentheses: A5 (D13395), SA11 (M23188), CMH222 (DQ288661), L26 (M58292), UK (M22306), Gottfried (M33516), OSU (X13190), KU (M21014), K8 (D90260), 69M (M60600), B223 (D13394), H-2 (L04638), MDR-13 (L07886), PA169 (D14724), Lp14 (L11599), EW (U08429), 993/83 (D16352), L338 (D13399), Mc323 (D38052), EHP (U08424), Hg18 (AF237665), 160/01 (AF526374), A34 (AY174094), TUCH (AY596189), Dhaka6 (AY773004), 134/04-15 (DQ061035).

[a] Amino acid identity was calculated based on VP8* region of VP4 gene.

Please cite this article as: Khamrin, P. et al. Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology (2006), doi:10.1016/j.virol.2006.12.004

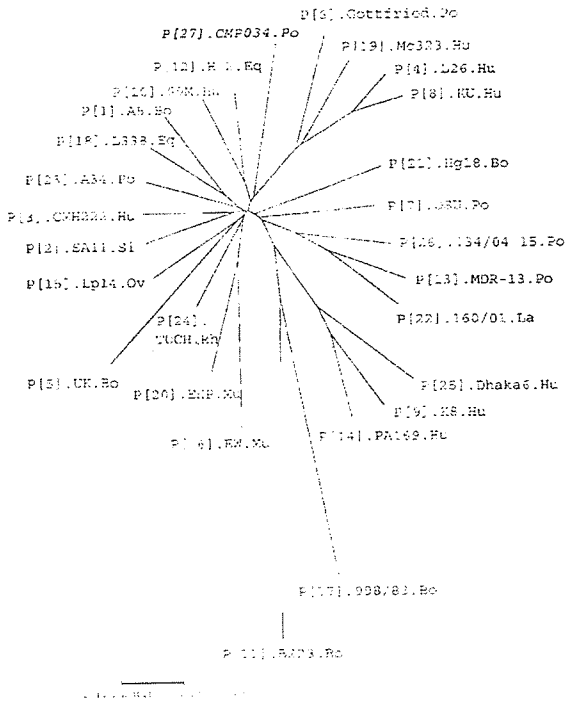

Fig. 1. Phylogenetic tree of the VP4 gene nucleotide sequence displaying the relationships between PoRV CMP034 and 26 known P genotypes. GenBank accession numbers of the VP4 sequences of all known 26 P genotypes are given in the legend to Table 1. The tree was generated on the basis of the neighbor-joining method using program MEGA 3.1.

PoRV strain CMP034 was located in a separate branch, which was only distantly related to the other P genotypes. The VP4 deduced amino acid sequence alignment of CMP034 with the representative rotavirus strains of all other P genotypes is shown in Fig. 2. The overall picture from this alignment showed the distinction between the CMP034 amino acid sequence and those of other representative strains of P[1] to P[26] genotypes. It was interesting to note that the complete deduced amino acid sequence of PoRV strain CMP034 had lost one amino acid residue at position 135, which was a unique feature of human, but not animal, rotavirus strains (Gorziglia et al., 1988; Kantharidis et al., 1987). However, the deletion of 3 amino acids observed in the hypervariable region of the VP5* portion of VP4 could also be found in bovine rotavirus strain B223 (Fig. 2). Accordingly, the origin of the CMP034 VP4 gene remained inconclusive. Furthermore, the potential trypsin cleavage sites at arginine 240 and 246 were conserved. The proline residues 68, 71, 224, 225, 333, 389, 394, 434, 450, 454, 474, 523, 665, 712, 745, and 757, and the cysteine residues at positions 215, 317, 379, which were highly conserved among the VP4 gene of rotaviruses, were maintained in the PoRV strain CMP034.

VP7 sequence analysis and determination of G genotype

Similar to the P genotype, the G genotype of PoRV strain CMP034 could not be determined by multiplex-PCR using the pools of G typing primer sets reported previously (Das et al., 1994; Gouvea et al., 1990). The full length of the nucleotide and deduced amino acid sequences of gene VP7, generated from RT-PCR, was determined and compared with VP7 sequences of the existing G1 to G15 strains. The highest sequence identity was found in G2 rotavirus strains (81.3–90.9% at the nucleotide level and 87.7–94.7% at the amino acid level), and showed the highest level of identity with PoRV G2-like reference strain 34461-4, 90.9% at the nucleotide level and 94.7% at the amino acid level (Table 2). Comparison between the VP7 sequences of PoRV CMP034 and human genotype G2 strains (DS-1, S2, TA20, KUN, 906SB/98, 92C) revealed nucleotide and amino acid identities ranging from 81.3 to 83.5% and from 87.7 to 88.0%, respectively.

In order to determine whether the failure of G genotyping of CMP034 was due to nucleotide mismatches at the primer binding region, the VP7 sequence of CMP034 was aligned with the sequences of G2 type-specific primers used in this study, aCT2 and 9T1-2 (Das et al., 1994; Gouvea et al., 1990). Several nucleotide mismatches were detected at the primer binding sites on VP7 gene of CMP034. The nucleotide mismatches were detected at 11 of 25 nucleotides for the aCT2 primer and at 5 of 20 nucleotides for the 9T1-2 primer, respectively (data not shown).

The amino acid sequences of VP7 antigenic regions A, B, C, and F of 15 established rotavirus G genotypes, as well as several G2 strains of human and porcine rotaviruses, were aligned with the respective sequence of the PoRV strain CMP034 (Fig. 3). Within the VP7 antigenic regions A, B, C, and F, PoRV strain CMP034 was identical with the G2 PoRV strain 34461-4, with the exception of one amino acid change from Lys to Arg in antigenic region B at position 147. In contrast, when comparing with human G2 reference strains (92C, 906SB/98, S2, TA20, KUN, and DS-1), a higher number of amino acid substitutions were found: 2 or 3 in antigenic regions A and C, 1 or 2 in antigenic region B, and 1 in antigenic region F. Thus, analysis of VP7 hypervariable regions A, B, C, and F confirmed the closest relationship of the PoRV strain CMP034 with G2-like PoRV strain 34461-4. A phylogenetic tree that included the VP7 sequences of all rotavirus G genotypes recognized to date from both human and non-human origins was constructed (Fig. 4). The result of phylogenetic analysis confirmed that PoRV CMP034 strain clustered with G2 rotavirus reference strains. Two major lineages were found among G2 rotavirus strains; one included most of the human G2 rotaviruses, including 92C, 906SB/98, S2, TA20, KUN, and DS-1, while another was formed exclusively by G2-like porcine rotavirus strains 34461-4 and CMP034.

VP6, NSP4, and NSP5/6 sequence analysis

Comparative analysis of the nucleotide and deduced amino acid sequences of full-length VP6 with those of four representative established subgroups allowed the identification of PoRV strain CMP034 as having subgroup I specificity. In addition, VP6 of strain CMP034 showed the highest level of nucleotide sequence identity (92.6%) with PoRV strain JL94, whereas the amino acid sequence is related most closely (99.2%) to that of PoRV strains A253 and A131 (Table 3). Strains JL94, A253, and A131 all have subgroup I specificity.

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Analysis of the NSP4 sequences revealed that CMP034 was related most closely to human rotavirus NSP4 genetic group B strain GR856/86, with 86.8% nucleotide sequence identity, and to strain RV4, with 88.2% amino acid sequence identity (Table 4). These results indicate that CMP034 belongs to NSP4 genetic group B.

The complete nucleotide sequence (667 nucleotides) of NSP5/6 gene of CMP034 was analyzed. By comparison to sequences in the GenBank database, the highest level of sequence identity (98.0% nucleotide identity) was with PoRV strain YM (data not shown).

Discussion

Several review articles have described the overview concerning the genetic and antigenic diversities of rotavirus

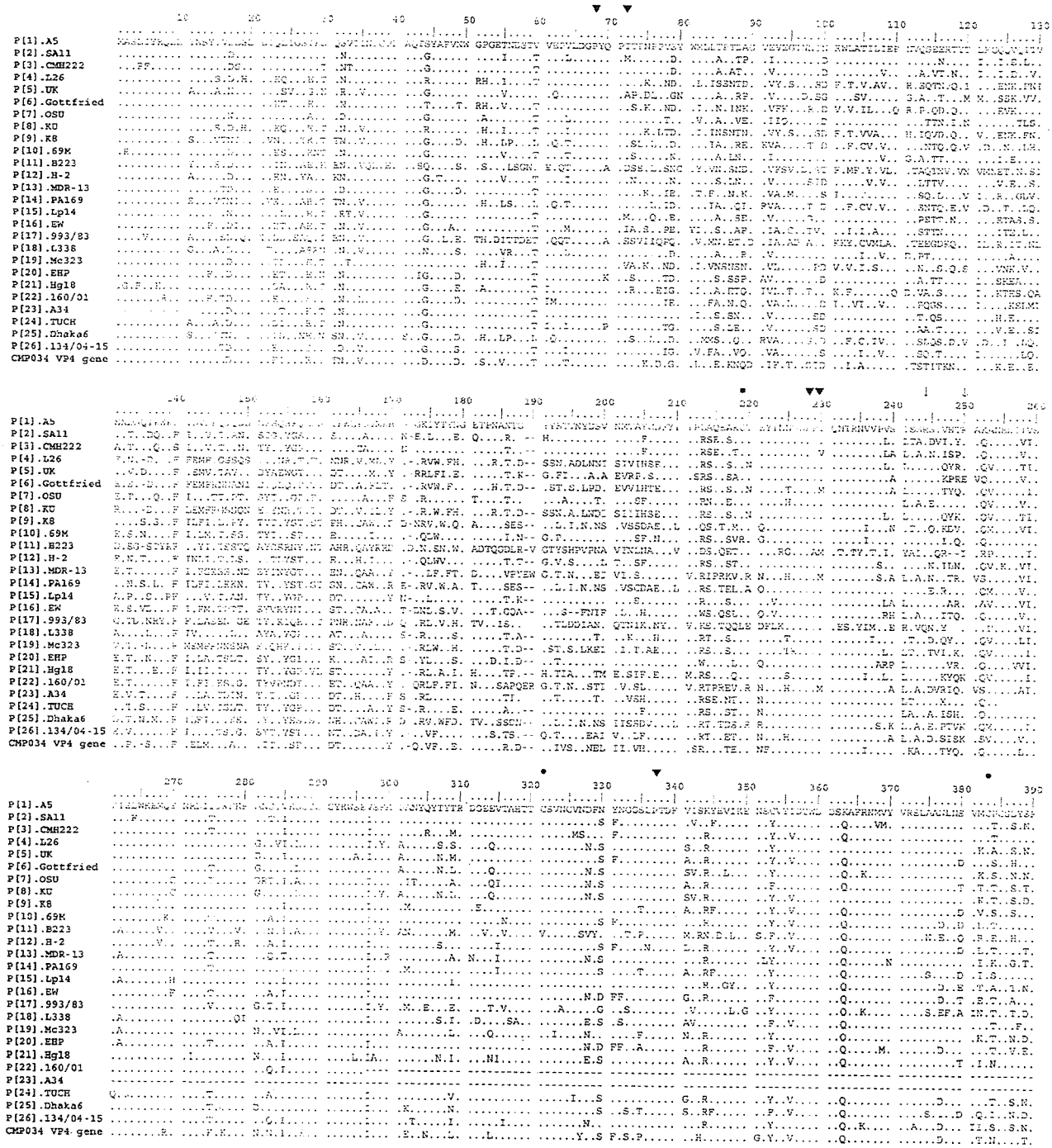

Fig. 2. Comparison of the deduced amino acid sequence of protein VP4 of the porcine rotavirus strain CMP034 with those of 26 known P genotypes. The potential trypsin cleavage site (↓) and highly conserved cysteine (●) and proline (▼) residues are indicated.

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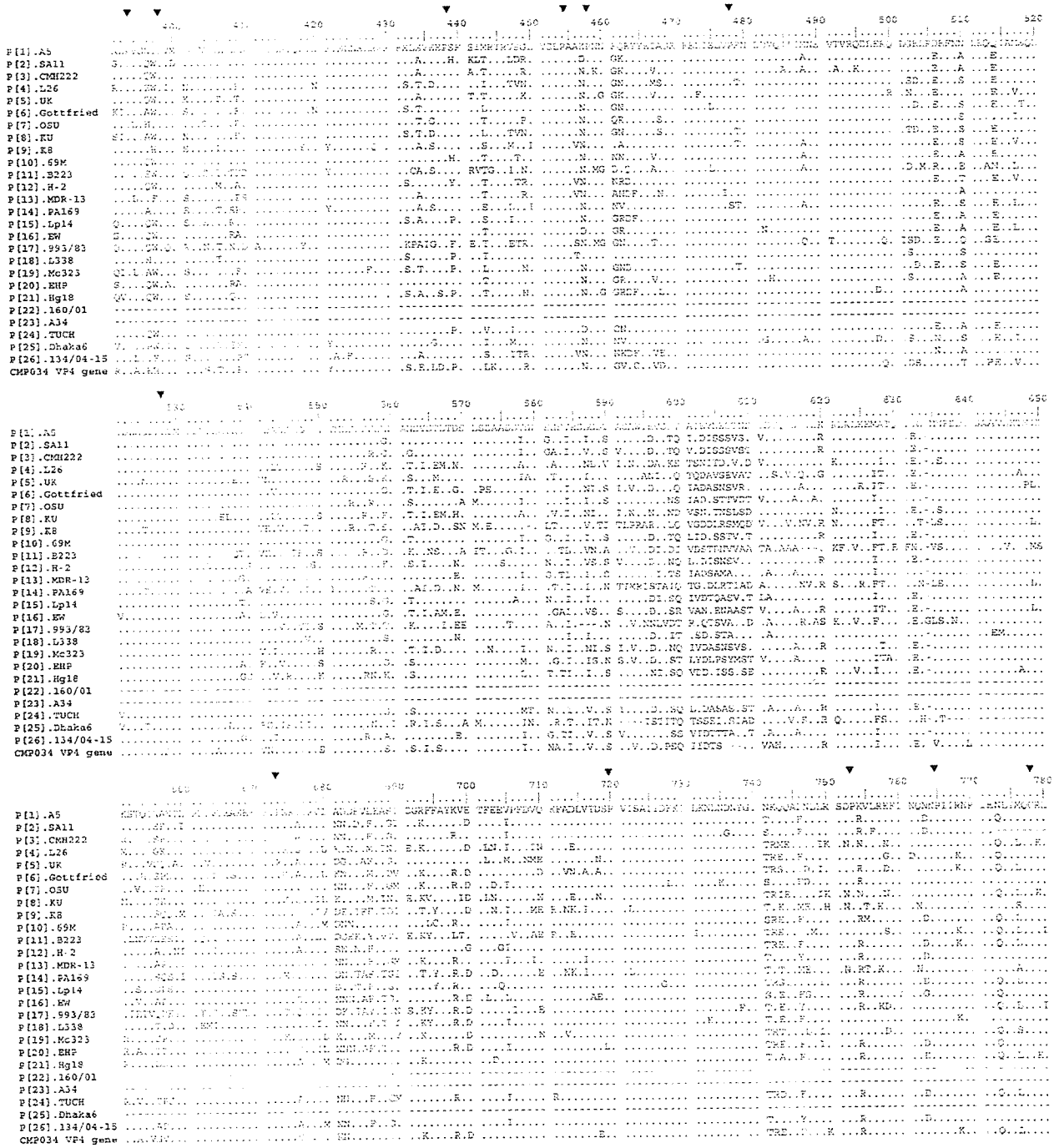

Fig. 2 (*continued*).

strains, and showed the potential evidence of close genetic relationships among rotavirus strains isolated from domestic animals and human host as a consequence of interspecies transmission (De Leener et al., 2004; Gentsch et al., 2005; Nakagomi et al., 1990; Palombo, 2002;). Therefore, simultaneous surveillance of animal and human rotavirus infections is a key step to understanding the ecology and evolution of rotaviruses.

Detection of human rotavirus strains bearing P[19] specificity (Mc323 and Mc345) was first reported from Chiang Mai city, Thailand in 1987–1989 (Urasawa et al., 1992). Analysis of the VP4 and VP7 sequences of these two strains revealed a G9P[19] specificity and close genetic relatedness to P[19] porcine rotavirus strain 4F (Okada et al., 2000). In that study, unfortunately, the epidemiological survey of porcine rotavirus was not done simultaneously in the same epidemic

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Table 2
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP7 of strain CMP034 with those of 15 known G rotavirus genotypes

Strain	Species	G genotype	Identity (%)	
			Nucleotide	Amino acid
KU	Human	G1	72.4	75.1
DS-1	Human	G2	81.3	88.0
S2	Human	G2	82.6	87.7
TA20	Human	G2	82.9	88.0
KUN	Human	G2	82.0	88.0
906SB/98	Human	G2	83.0	88.0
92C	Human	G2	83.5	88.0
34461-4	Porcine	G2	90.9	94.7
CMH222	Human	G3	73.6	76.6
Hochi	Human	G4	72.5	72.3
OSU	Porcine	G5	74.1	76.0
NCDV	Bovine	G6	72.3	75.7
Ch2	Avian	G7	61.0	56.9
B37	Human	G8	73.5	57.1
116E	Human	G9	77.0	77.3
61A	Bovine	G10	71.7	74.2
YM	Porcine	G11	72.9	75.1
L26	Human	G12	74.8	75.1
L338	Equine	G13	72.0	72.6
CH3	Equine	G14	73.5	73.9
Hg18	Bovine	G15	72.1	72.3

The GenBank accession numbers of the following strains are given in parentheses: KU (D16343), DS-1 (AB118023), S2 (M11164), TA20 (AF106281), KUN (D50124), 906SB/98 (AY261347), 92C (U73949), 34461-4 (AY766085), CMH222 (AY707792), Hochi (AB012078), OSU (X04613), NCDV (M12394), Ch2 (X56784), B37 (J04334), 116E (L14072), 61A (X53403), YM (M23194), L26 (M58290), L338 (D13549), CH3 (D25229), Hg18 (AF237666).

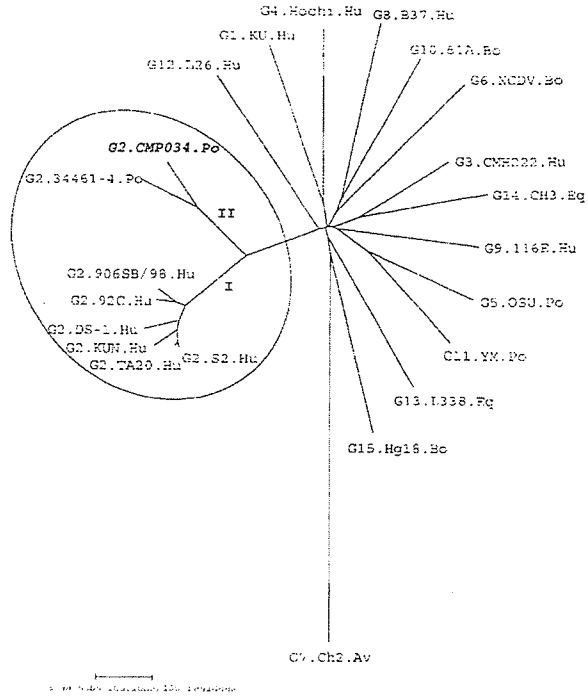

Fig. 4. Phylogenetic tree based on the VP7 nucleotide sequences. The tree displays the relationships among strain CMP034 and of the 15 known G genotypes. GenBank accession numbers of the VP7 sequences of all known 15 G genotypes are given in the legend to Table 2. The tree was generated on the basis of the neighbor-joining method using program MEGA 3.1.

season to verify whether P[19] was really circulating in the pig population in Chiang Mai city. Therefore, we conducted a rotavirus surveillance in both humans and pigs simultaneously in the same epidemic season in Chiang Mai city during the year 2000–2001, and we found P[19] strains circulating in pig populations in Chiang Mai city (Maneekarn et al., 2006). These PoRV strains were G3P[19], and shared a level of VP4 P [19] sequence identity with those of P[19] Mc323 and Mc345 as high as 95.4–97.6% nucleotide sequence identity. This finding indicates that gene VP4 of Mc323 and Mc345 might have been derived through reassortment from VP4 P[19] of PoRV strains that circulated in this region. However, P[19] rotavirus was not detected in humans in the study that was carried out in the same epidemic season (Khamrin et al.,

	A		B		C		F	
	87	161	142	162	208	221	235	240
G2.CMP034	TYVPTEAKNEISDTE		NVVLMRYDTTS		GIGCKTTEVNTFET		NGVNHKTS	
G2.34461-4	.....		.....K.....		.....		.....	
G2.92C	L.....	.D.	.....	.N..	.....D.S...I		.....N	
G2.906SB/98	L...A.....	.D.	.....	.GN..	.....D.S....		.....N	
G2.S2	L...A.....	.D.	.....	.N..	.....D.D...I		.....N	
G2.TA20	L...A.....	.D.	.....	.N..	.....D.D...I		.....N	
G2.KUN	L.....	.D.	.....	.N..	.....D.....I		.....N	
G2.DS-1	L...A.....	.D.	.....	.N..	.....D.....I		.....N	
G1.KU	L.....SPQ.N.GD		.L...K..QSL		....Q..N.DS..M		D.I....N	
G3.CMH222	L.....AT..N.NS		.....K..A.L		....L..DTT...E		D.....LD	
G4.Hochi	L...S..PTQ.....		....I.PASGE		....Q..NTA....		DS....LD	
G5.OSU	L...N..AT..A..K		.I...K..GNL		....S..DI.S...		D.....LD	
G6.NCDV	L...V..S..MA...		.L...K..S.Q		....LI.NPD....		D.....LN	
G7.Ch2	....KA.DT..A.P.		.I..AH.TNDV		....Q..NTD...I		D.....VD	
G8.B37	L...V..ET..A.SS		.I...K.NAN.		R...L..DTT...E		D...Y..N	
G9.116E	L...T..STQ.G...		....VK.NS.L		....T..NTA...E		D.....LD	
G10.61A	L.....RT..N.N.		.I.....NSSL		....Q..NTR...E		D.....LD	
G11.YM	L...H..ATQ.A.DK		.I...K..GN.		....L..DPT...E		D.....LD	
G12.L26	L...NSVTT..T.PD		.I..VQ.QNSL		....T..D.A...E		D.....N	
G13.L338	L...N.VVS.LN.DS		.I.VVK.S.EL		....L..DTE...E		D.....N	
G14.CH3	L.S....ATQ.D.SS		.....K..EAL		....L..N.E...E		D..D...N	
G15.Hg18	.....I.S.DLA.PD		.I...K.ESDL		....L..DTSS...		D..S..LD	

Fig. 3. Comparison of the amino acid sequences of antigenic regions A, B, C, and F of porcine CMP034 with those of 15 known G genotypes. Dots indicate amino acid residues identical with those in the sequence of strain CMP034.

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Table 3
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein VP6 of strain CMP034 with those of known subgroups

Strain	Species	Subgroup	Identity (%)	
			Nucleotide	Amino acid
BRV033	Bovine	I	76.8	90.1
NCDV	Bovine	I	79.1	90.9
UK	Bovine	I	80.0	91.4
SA11	Simian	I	78.7	91.1
I321	Human	I	80.7	90.9
US1205	Human	I	78.6	90.1
1076	Human	I	79.7	91.6
RMC321	Human	I	90.8	98.9
RMC/G60	Human	I	90.7	98.4
RMC/G7	Human	I	90.9	98.4
4F	Porcine	I	91.0	98.9
4S	Porcine	I	90.9	98.9
JL94	Porcine	I	92.6	98.4
A253	Porcine	I	86.0	99.2
A131	Porcine	I	87.5	99.2
OSU	Porcine	I	86.9	98.9
H-1	Equine	I	87.7	97.9
Wa	Human	II	82.1	93.7
116E	Human	II	83.4	93.9
TK159	Human	II	82.9	91.9
Gottfried	Porcine	II	83.6	93.7
FI-14	Equine	I+II	78.9	91.1
L338	Equine	I+II	80.5	92.1
H-2	Equine	Noni/nonII	78.9	90.2
FI-23	Equine	Noni/nonII	78.5	90.2

The GenBank accession numbers of the following strains are given in parentheses: BRV033 (AF317126), NCDV (AF317127), UK (X53667), SA11 (AY187029), I321 (X94618), US1205 (AF079357), 1076 (D00325), RMC321 (AF531913), RMC/G60 (AY601552), RMC/G7 (AY601551), 4F (L29184), 4S (L29186), JL94 (AY538664), A253 (AF317122), A131 (AF317124), OSU (AF317123), H-1 (AF242394), Wa (K02086), 116E (U85998), TK159 (AY661888), Gottfried (D00326), FI-14 (D00323), L338 (D82974), H-2 (D00324), FI-23 (D82971).

2006a). In this epidemiological study of the distribution of PoRV G and P genotypes in Chiang Mai province, we detected an unusual rotavirus strain, which appears to carry a novel P[27] genotype. As revealed by the analysis of the amino acid sequence, gene VP4 of PoRV strain CMP034 shares less than 77% amino acid sequence identity with the 26 known P genotypes.

It is well established that the VP4 sequence of animal rotaviruses contains 776 amino acid residues, while most human rotavirus strains contain 775 residues, with the loss of one residue between positions 134 and 136 of the VP8* fragment of gene VP4 (Gorziglia et al., 1988; Kantharidis et al., 1987). Analysis of the amino acid sequence of VP4 revealed that CMP034 has lost one amino acid residue at position 135, similar to human rotaviruses. Nevertheless, the deletion of three other amino acid residues in the hypervariable region of the VP5* portion of gene VP4, like those found in bovine rotavirus strain B223, implies an animal origin of CMP034. This unusual feature of amino acid deletion in the VP4 gene of CMP034, therefore, makes the origin of gene VP4 unclear. Nevertheless, it is interesting to note that sequence analyses of VP6 and NSP5/6 genes of CMP034 reveal a porcine-like specificity, whereas the NSP4 gene has some characteristics consistent with a human origin.

The distribution of rotavirus G and P genotypes in pigs has been reported from throughout the world (Estes, 2001; Liprandi et al., 1991; Pongsuwanna et al., 1996; Winiarczyk et al., 2002). On the basis of the accumulated evidence of transmission of rotaviruses between pigs and other animal species, including human, pigs are regarded as the potential reservoir for the emergence of unusual or novel strains of rotaviruses (Palombo, 2002; Martella et al., 2005, 2006b). Several novel strains of P genotypes have been identified recently in pigs, such as P[23] (Liprandi et al., 2003) and, most recently, P[26] (Martella et al., 2006a). In the present study, a novel P genotype has been isolated from a diarrheic piglet in Thailand.

Although G2 strains of rotavirus are commonly found in humans, it has not been identified from other animal sources (Estes, 2001), except for one recent report from Spain of a

Table 4
Comparison of the nucleotide and amino acid sequence identities of the genome segment encoding protein NSP4 of strain CMP034 with those of known NSP4 genetic groups

Strain	Species	NSP4 genetic group	Identity (%)	
			Nucleotide	Amino acid
KUN	Human	A	78.6	78.8
UK	Bovine	A	79.0	80.0
NCDV	Bovine	A	78.1	80.0
SA-11	Simian	A	78.9	78.2
Wa	Human	B	85.1	84.0
RV4	Human	B	84.5	88.2
ST3	Human	B	83.1	81.1
M37	Human	B	83.9	81.7
116E	Human	B	85.9	83.4
97'SZ37	Human	B	85.4	83.4
GR856/86	Human	B	86.8	86.2
GR1106/86	Human	B	86.6	85.1
A131	Porcine	B	79.8	85.4
A253	Porcine	B	81.8	85.7
A411	Porcine	B	81.2	82.8
H-1	Equine	B	81.9	83.4
OSU	Porcine	B	85.7	85.1
A34	Porcine	B	86.0	85.1
FRV-1	Feline	C	77.8	77.1
AU1	Human	C	77.9	76.5
GRV	Caprine	C	61.0	78.2
CMH222	Human	C	78.0	77.1
CU-1	Canine	C	73.3	77.7
EW	Murine	D	66.2	60.0
EHP	Murine	D	66.1	59.4
EC	Murine	D	66.0	58.2
Ty-1	Avian	E	49.8	30.2
Ty-3	Avian	E	46.8	29.7
Ch-1	Avian	E	51.3	32.5

The GenBank accession numbers of the following strains are given in parentheses: KUN (D88829), UK (K03384), NCDV (X06806), SA11 (K01138), Wa (AF093199), RV4 (U59108), ST3 (U59110), M37 (U59109), 116E (U78558), 97'SZ37 (AF284778), GR856/86 (AF170832), GR1106/86 (AF170833), A131 (AF144798), A253 (AF144797), A411 (AF144799), H-1 (AF144800), OSU (D88831), A34 (AF165219), FRV-1 (D89874), AU-1 (D89873), GRV (AB055968), CMH222 (DQ288660), CU-1 (AF144806), EW (U96335), EHP (U96336), EC (U96337), Ty-1 (AB065285), Ty-3 (AB065286), Ch-1 (AB065287).

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PoRV strain bearing a G2-like genotype (strain 34461-4) in a piglet (Martella et al., 2005). The VP7 sequence analysis of our CMP034 strain revealed a genetically close relationship to the G2-like PoRV strain 34461-4. A close genetic relationship between VP7 from strains CMP034 and 34461-4 was demonstrated by analysis of the VP7 antigenic regions A, B, C, and F. The only difference observed between the two strains was a Lys to Arg change at position 147 in the antigenic region B. The relatedness between the sequence of VP7 in strains CMP034 and 34461-4 was confirmed repeatedly by the phylogenetic analysis of the VP7 sequence, showing that CMP034 and 34416-4 are clustered closely together in a branch separate from that of other human G2 reference strains. These findings suggest that VP7 of PoRV strain CMP034 may have originated from the same ancestor as those of strain 34461-4. The isolation of two strains of rotaviruses with a close genetic relatedness of gene VP7 from two countries, Thailand and Spain, which are geographically far apart, may indicate that the gene VP7 of G2 specificity may have already been introduced into the porcine rotaviruses worldwide. To verify this notion, the epidemiological rotavirus surveillance in pigs may need to be performed extensively in several other regions of the world.

Materials and methods

Rotavirus antigen detection

Porcine rotavirus strain CMP034 was isolated during the surveillance of porcine rotavirus infection in Chiang Mai province, Thailand from June 2000 to July 2001. A total of 175 fecal specimens were collected from diarrheic piglets from six different farms and the age of the piglets ranged from 7 days to 49 days old. Group A rotavirus antigen was detected by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody against group A rotavirus as described previously (Hasegawa et al., 1987). Out of 175, 39 (22.3%) were positive for group A rotaviruses (Maneekarn et al., 2006). PoRV strain CMP034 was identified as group A rotavirus in stool sample from a diarrheic piglet of 49 days old at a farm in Mae Rim district, Chiang Mai province. Despite numerous attempts, using RNA PAGE of phenol/chloroform or acid phenol/guanidinium thiocyanate/chloroform RNA extraction methods, we were unable to visualize the dsRNA electrophoretic pattern.

RNA extraction, RT-PCR, and multiplex-PCR for G and P genotyping

The G and P genotypes of CMP034 were determined by RT-PCR and multiplex-PCR. Viral dsRNA was extracted from 10% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). Viral dsRNA was denatured in 50% (v/v) dimethyl sulfoxide at 95 °C for 5 min. The RT-PCR was carried out with a OneStep RT-PCR Kit (Qiagen, Hilden, Germany). For PCR amplification of the VP7 gene, a 1062 bp fragment was generated using Beg9 (forward) and End9 (reverse) primers (Gouvea et al., 1990). For PCR amplification of the VP4 gene, an 876 bp fragment was generated using Con3 as a forward primer and Con2 as a reverse primer (Gentsch et al., 1992). The G genotyping was performed using different pools of primers specific for G genotypes of human and animal rotaviruses (G1–G6 and G8–G11) as described previously (Das et al., 1994; Gouvea et al., 1990, 1994a; Winiarczyk et al., 2002). The VP4 characterization was performed using different pools of P genotype-specific primers for P[1], P[4]-P[11], and P[14] (Gentsch et al., 1992; Gouvea et al., 1994b; Mphahlele et al., 1999; Winiarczyk et al., 2002).

Amplification and sequence analysis of VP4, VP7, VP6, NSP4, and NSP5/6 genes

We could not initially identify the P genotype of PoRV CMP034 strain by multiplex PCR using several sets of genotype-specific primers, so the reverse primer 170 (Martella et al., 2006a), was used in combination with Con3 (forward primer) for amplification of gene VP4 (2341 bp). The full-length VP7 gene (1062 bp) was reverse transcribed and amplified using the primer pair of Beg9 and End9 (Gouvea et al., 1990). The G genotype of CMP034 strain was not

Table 5
Oligonucleotide primers used for the amplification and sequencing of genes VP4, VP6, VP7, NSP4, and NSP5/6

Primer	Gene	Sequence 5′ to 3′	Sense	Position	Reference
Con3	VP4	TGGCTTCGCTCATTTATAGACA	+	11–32	Gentsch et al. (1992)
170	VP4	GGTCACAWCCTCTAGMMRYTRCTTA	–	2362–2383	Martella et al. (2006a)
Con2	VP4	ATTTCGGACCATTTATAACC	–	868–887	Gentsch et al. (1992)
Con2R[a]	VP4	GGTTATAAATGGTCCGAAAT	+	868–887	Gentsch et al. (1992)
VP4-3R	VP4	CAATTCTRTTHCGAATTATTGGRTT	–	2287–2311	Khamrin et al. (2006a)
P34F665	VP4	GATTGCCACCAATACAGAAC	+	665–684	This study
P34F2089	VP4	GAGTAGACACGTTTGAGGAGG	+	2069–2089	This study
Beg9	VP7	GGCTTTAAAAGAGAGAATTTCCGTCTGG	+	1–28	Gouvea et al. (1990)
End9	VP7	GGTCACATCATACAATTCTAATCTAAG	–	1036–1062	Gouvea et al. (1990)
VP6-5F	VP6	GGCTTTTAAACGAAGTCTTC	+	1–20	Shen et al. (1994)
VP6-3R	VP6	GGTCACATCCTCTCACTA	–	1339–1356	Shen et al. (1994)
NSP4 1a	NSP4	GGCTTTTAAAAGTTCTGTTCCG	+	1–22	Kudo et al. (2001)
NSP4 2b	NSP4	GGTCACATTAAGACCGTTCC	–	731–750	Kudo et al. (2001)
GEN-NSP5F	NSP5/6	GGCTTTTAAAGCGCTACAG	+	1–24	Matthijnssens et al. (2006)
GEN-NSP5R	NSP5/6	GGTCACAAAACGGGAGTG	–	650–667	Matthijnssens et al. (2006)

[a] Con2R primer was modified from the original Con2 primer described by Gentsch et al. (1992).

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determined, even by using several sets of genotype-specific primers. In order to determine the G genotype specificity, we analyzed the sequence of gene VP7. The full length of gene VP6 was amplified by primer pairs VP6-5F and VP6-3R, which were modified slightly from the original VP6-specific primers described by Shen et al. (1994). The NSP4 full-length gene was amplified by the NSP4-1a and NSP4-2b primer pair (Kudo et al., 2001). The full-length NSP5/6 gene was amplified with the pair of primers, GEN-NSP5F and GEN-NSP5R, described by Matthijnssens et al. (2006). The sequences of primers used for amplification and sequencing are shown in Table 5.

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). The nucleotide and deduced amino acid sequences of genes VP4, VP7, VP6, NSP4, and NSP5/6 were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) server (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.1 (Kumar et al., 2004).

Nucleotide sequence accession number

The nucleotide sequences of genes VP4, VP6, VP7, NSP4, and NSP5/6 of strain CMP034 have been deposited in GenBank with the accession numbers DQ534016, DQ534018, DQ534015, DQ534017, and DQ916134, respectively.

Acknowledgments

This research was supported by the Japan Society for the Promotion of Science (JSPS) under the NRCT-JSPS project. The study was supported also, in part, by the Endowment Fund for Medical Research, Faculty of Medicine, Chiang Mai University, Thailand.

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Please cite this article as: Khamrin, P. et al. Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology (2006), doi:10.1016/j.virol.2006.12.004

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Please cite this article as: Khamrin, P. et al. Novel porcine rotavirus of genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology (2006), doi:10.1016/j.virol.2006.12.004

JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2006, p. 4113–4119 Vol. 44, No. 11
0095-1137/06/$08.00+0 doi:10.1128/JCM.00954-06

Detection of Rare G3P[19] Porcine Rotavirus Strains in Chiang Mai, Thailand, Provides Evidence for Origin of the VP4 Genes of Mc323 and Mc345 Human Rotaviruses[▽]

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Received 7 May 2006/Returned for modification 7 July 2006/Accepted 13 September 2006

Among 175 fecal specimens collected from diarrheic piglets during a surveillance of porcine rotavirus (PoRV) strains in Chiang Mai, Thailand, 39 (22.3%) were positive for group A rotaviruses. Of these, 33.3% (13 of 39) belonged to G3P[19], which was a rare P genotype seldom reported. Interestingly, their VP4 nucleotide sequences were most closely related to human P[19] strains (Mc323 and Mc345) isolated in 1989 from the same geographical area where these PoRV strains were isolated. These P[19] PoRV strains were also closely related to another human P[19] strain (RMC321), isolated from India in 1990. The VP4 sequence identities with human P[19] were 95.4% to 97.4%, while those to a porcine P[19] strain (4F) were only 87.6 to 89.1%. Phylogenetic analysis of the VP4 gene revealed that PoRV P[19] strains clustered with human P[19] strains in a monophyletic branch separated from strain 4F. Analysis of the VP7 gene confirmed that these strains belonged to the G3 genotype and shared 97.7% to 98.3% nucleotide identities with other G3 PoRV strains circulating in the regions. This close genetic relationship was also reflected in the phylogenetic analysis of their VP7 genes. Altogether, the findings provided peculiar evidence that supported the porcine origin of VP4 genes of Mc323 and Mc345 human rotaviruses.

Rotavirus is the major cause of acute gastroenteritis in infants and young children and in young animals of a large variety of species (27). It contains two outer capsid proteins, VP7 and VP4, which independently elicit neutralizing antibodies and specify the G and P genotypes of the virus, respectively (14, 16, 24, 27, 40). To date, 15 distinct G genotypes and 26 P genotypes have been identified (14, 27, 30, 31, 32, 33, 35, 46, 48). Epidemiological studies of porcine rotaviruses (PoRV) in several countries have identified at least four main G types, G3, G4, G5, and G11, which are the most common (14). However, other porcine rotaviruses, such as G1, G2, G6, G8, G9, and G10, have also been reported occasionally (1, 5, 6, 19, 25, 32, 34, 43, 45, 52, 57). For the P type, P[6] and P[7] were found to be the most common genotypes in pigs, while other P types, such as P[13], P[14], P[19], P[23], and P[26], were seldom reported (3, 14, 20, 25, 27, 30, 31, 59). In Thailand, the epidemiological study of the group A rotavirus in pigs has been limited, and G3 had been the only G type detected in the last decade (44), until G10 was recently reported (43). Rotaviruses belonging to the same G serotype usually share at least 90% amino acid sequence identity (21), whereas viruses of the same P genotype normally share more than 89% amino acid sequence identity (4, 14, 16). The increased detection of rotavirus strains bearing an unusual combination of phenotypes of human and animal rotaviruses has been well documented (10, 12, 42, 47). This observation supports the hypothesis that interspecies transmission of rotaviruses from one animal species to others, including humans, might take place in nature (7, 18, 37, 42). The interspecies transmission could be the result of infection with an animal rotavirus virion (38) or could occur via genetic reassortment between humans and animal rotavirus strains during coinfection of the same cell (9, 11, 54, 55).

Two strains of human G9 rotaviruses (Mc323 and Mc345) isolated in 1989 in Chiang Mai, Thailand, had been shown by RNA-RNA hybridization to be more closely related to the porcine G9 rotavirus than to human rotaviruses (54). Recently, analyses of VP7 and VP4 nucleotide and deduced amino acid sequences of Mc323 and Mc345 revealed that both strains belonged to G9P[19] genotype (41), with the VP7 sequences closely related to the G9 human rotaviruses WI61 and F45, while the VP4 sequence revealed a close genetic relatedness to that of the P[19] porcine rotavirus 4F reported previously (2). Most recently, a human rotavirus G9P[19] isolate (RMC321) with porcine rotavirus characteristics was also reported following an outbreak of infantile gastroenteritis in India (55). Currently, G9 is a common genotype of humans and pigs (32), while P[19] is a rare one in both of them (31). It is possible that human G9P[19] strains Mc323 and Mc345 might have arisen by natural reassortment among rotavirus strains of human and porcine origins that circulated in the Chiang Mai area. Unfortunately, in that study, the rotavirus strain surveillance in pigs was not carried out simultaneously. It is, therefore, tempting to verify whether the P[19] rotavirus, a rare genotype, is really circulating in the porcine population of the Chiang Mai area.

In this study, 13 strains of G3P[19] PoRV were isolated from diarrheic piglets raised in several pig farms located in Chiang

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▽ Published ahead of print on 20 September 2006.

Mai, Thailand. The VP4 genes of these strains were most closely related to those of Mc323 and Mc345, the human rotaviruses that were also isolated from Chiang Mai. These findings provided evidence that supported the porcine origin of VP4 genes of Mc323 and Mc345 strains.

MATERIALS AND METHODS

Specimen collection. A total of 175 fecal specimens were collected from diarrheic piglets from six different farms located in Chiang Mai province, Thailand. The ages of the piglets ranged from 7 to 49 days old. The specimens were collected from June 2000 to July 2001 and stored at −20°C until use.

Screening and subgrouping of the group A rotavirus by ELISA. The presence of the group A rotavirus in fecal specimens was detected by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody against it, as described previously (22). The subgroup specificities of the virus were determined by ELISA using monoclonal antibodies (MAbs) specific to subgroup I and subgroup II rotaviruses (Serotec, Japan), as described previously (53).

RNA extraction and polyacrylamide gel electropherotyping. Viral genomic RNA was extracted from fecal specimens by use of a phenol-chloroform (22) or an acid phenol-guanidinium thiocyanate-chloroform (51) extraction method. The extract was subjected to polyacrylamide gel electrophoresis for the detection of viral genomic RNA and characterization of RNA electrophoretic pattern, as described previously (49). The localization of RNA genome fragments migrated in the gel was detected by silver staining, as described previously (23).

G genotyping. The G genotype was determined by using a protocol modified from a method previously described (17). Briefly, the RNA genome of the rotavirus was first extracted from 10% fecal supernatant by use of a QIAamp viral RNA mini kit (QIAGEN). Reverse transcription-PCR (RT-PCR) was performed using a OneStep RT-PCR kit (QIAGEN). The full length of the VP7 gene was reverse transcribed and simultaneously amplified by using Beg9 and End9 primers in a single tube reaction. The expected size of the PCR product generated from the full-length VP7 gene was 1,062 bp in length. The second amplification was performed using the first PCR product as the template together with G-genotype-specific mixed primers BT1, CT2, ET3, DT4, FT8, and FT9 for upstream priming and the End9 primer for downstream priming of VP7 genes for identifications of genotypes G1 to G4, G8, and G9. The samples for which the G genotype could not be identified by the first set of primers described by Gouvea et al. (17) were later identified by using alternative sets of type-specific primers reported by Das et al. (8), Gouvea et al. (19), and Winiarczyk et al. (57). These primer sets covered a wide range of rotavirus genotypes, i.e., G1 to G4, G5, G6, and G8 to G11.

P genotyping. The P genotype was identified by using a method modified from that described by Gentsch et al. (15). Briefly, the partial sequence of the VP4 gene was reverse transcribed and simultaneously amplified by using Con2 and Con3 primers. In the second amplification, a mixture of primers 1T-1, 2T-1, 3T-1, 4T-1, 5T-1, and ND2 with Con3 primers was utilized for the identification of P[8], P[4], P[6], P[9], P[10], and P[11], respectively. The samples for which the P genotype could not be identified by the first set of type-specific primers were then genotyped by using alternative sets of type-specific primers, as previously reported (20, 36, 57). These primer sets were specific for P[1], P[4] to P[11], and P[14] genotypes.

Nucleotide sequence analysis. The rotavirus isolates of which the G or P genotypes could not be determined by type-specific primers were then subjected to nucleotide sequencing. The PCR products of VP7 or VP4 genes obtained from the first amplification of each nontypeable isolate were purified by a QIAquick PCR purification kit (QIAGEN) and then subjected to direct nucleotide sequencing according to the manufacturer's instructions by using a BigDye Terminator cycle sequencing kit (PE Biosystems). The nucleotide sequences were analyzed by comparison with those of the reference strains available in the GenBank database.

Design of the new typing primer for P[19] and PCR optimization. The VP4 nucleotide sequences of 13 isolates of the P[19] porcine rotavirus detected in this study, together with the sequences of other P[19] strains, such as Mc323, Mc345, and 4F, as well as other P types (P[1] to P[26]) selected from the GenBank database, were aligned using the ClustalX program. The region highly conserved among P[19] strains and that divergent in other P types were selected as a primer sequence. The newly designed primer for P[19], namely, VP4P19, was targeted to nucleotides (nt) 400 to 425 of the VP4 gene. The nucleotide positions and sequence of the primer (5′ to 3′) were as follows: AAC TTC CAY TTA YTT GAG GTA TTA AC (nt 400 to 425; Y = C or T). The VP4P19 primer (forward) was used in combination with the Con2 primer (reverse) (nt 868 to 887) in the second-round PCR to generate a 415-bp product.

The specificity of the VP4P19 primer was evaluated by testing the primer with all 13 isolates of P[19], three isolates of P[13], and one each of P[3], P[4], and P[12]. Briefly, 3 μl of RNA genome was added to 0.3 μl of 50% dimethyl sulfoxide before being heated at 97°C for 5 min and then rapidly cooled on ice. The denatured RNA was then reverse transcribed for 1 h at 37°C in 25 μl of reaction mixture containing 15.8 μl of RNase-free water and 2.5 μl of 10× PCR buffer containing 12.5 mM $MgCl_2$, 2.0 μl of 10 mM of each deoxynucleoside triphosphate mix (10 mM of each deoxynucleoside triphosphate), 0.4 μl of each (33-pmol/μl) primer pair of Con3 (forward) and Con2 (reverse), 0.5 μl of avian myeloblastosis virus-RT enzyme (200 U/μl), and 0.2 μl of RNase inhibitor. The cDNA was then amplified further with 0.5 μl of *Taq* DNA polymerase (5 U/μl) for 35 cycles, with a thermocycling condition at 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min and a final extension at 72°C for 7 min. The first PCR product was diluted at 1:100 and subjected to the second-round PCR, in which VP4P19 and Con2 were used as typing primers. The thermal cycling profile was 35 cycles of 94°C for 1 min, 45°C for 2 min, 72°C for 2 min, and a final extension at 72°C for 7 min. The second PCR product was detected by electrophoresis on 1.5% agarose gel in Tris-borate-EDTA buffer and stained with ethidium bromide. The P[19] genotype was identified based on the presence of the DNA band of a PCR product of 415 bp in length and confirmed by nucleotide sequence analysis.

Nucleotide sequence accession numbers. The nucleotide sequences of G3P[19] porcine rotavirus strains described in this study were deposited in the GenBank database. The accession numbers for the VP4 sequences of the 13 strains of porcine P[19] described in this study were as follows: for strain CMP029, accession no. AY689219; for strain CMP031, AY689218; for strain CMP039, AY689217; for strain CMP072, AY689216; for strain CMP087, AY689215; for strain CMP090, AY689214; for strain CMP092, AY689213; for strain CMP094, AY689212; for strain CMP095, AY689211; for strain CMP096, AY689210; for strain CMP098, AY689209; for strain CMP099, AY68208; and for strain CMP100, AY689207. The accession numbers for the VP7 sequences of the five representative strains of G3 described in this study were as follows: for strain CMP039, accession no. AY707788; for strain CMP096, DQ256502; for strain CMP099, DQ256503; for strain CMP213, DQ786576; and for strain CMP214, DQ786577.

RESULTS

Prevalence and characteristics of the porcine group A rotavirus. The group A rotavirus was detected in 39 out of 175 (22.3%) fecal specimens collected from diarrheic piglets in Chiang Mai, Thailand, from June 2000 to July 2001. The characteristics of the viruses are as follows. Among 39 isolates of the group A rotavirus, 8 isolates (20.5%) belonged to subgroup I (SG I), 2 isolates (5.1%) belonged to SG II, and 5 isolates (12.8%) showed a dual subgroup specificity, i.e., they were reactive to both MAbs specific for SG I and SG II. The majority of the virus isolates (24 out of 39 [61.6%]) were not reactive to MAbs specific for either SG I or SG II. The electrophoretic pattern of genomic viral RNA could be demonstrated in only 17 out of 39 (43.6%) fecal samples, and all of these isolates displayed a long electropherotype, while another 22 isolates (56.4%) were in a smear pattern. Therefore, their electropherotype could not be assigned by polyacrylamide gel electrophoresis. However, the RNA genome of these isolates could be amplified by RT-PCR using the consensus primers Con2 and Con3 for VP4 and Beg9 and End9 for VP7.

Distributions of G and P genotypes of the porcine rotavirus. Among 39 isolates of porcine group A rotaviruses, five different G genotypes, G2, G3, G4, G5, and G9, were detected in this study (Table 1). The G3 and G4 genotypes were copredominant genotypes, with a prevalence of 43.6% and 46.2%, respectively, followed by much less prevalent G5 (5.1%) and G2 (2.6%) genotypes. Most of the G4 genotypes (17 of 18 isolates) were found in combination with P[6], while the re-