(1, 2, 5, 6型) の感染で数ヶ月～数年に渡ってウイルスが糞便中に間歇的に排出される。したがって，C種アデノウイルスを糞便から検出した場合，その病原体としての意義は臨床症状等で総合的に慎重に判断する必要がある。しかし，F種アデノウイルスが糞便中から検出された場合は胃腸炎の起因病原体である可能性が高い。F種アデノウイルス抗原検出用ELISAキットでは，発症から6日以上経過した検体では陰性となる可能性があると記載されている。

検査法による検出感度は，我々の検討[3]ではPCR，リアルタイムPCR＞ウイルス分離＞ICキットの順に高かった。ELISAはICキットとほぼ同等の感度とされている。PCRおよびウイルス分離では，系によって検出感度に大きな差があるので，比較においては手法を明示すべきである。

アデノウイルスの迅速診断

アデノウイルスの迅速診断の意義として，

- アデノウイルスの院内感染・施設内感染の拡大防止に役立つ。
- アデノウイルス感染症は高いCRP値と高熱など，細菌感染症との鑑別が難しい場合があり，迅速診断によりアデノウイルスと同定できれば無駄な抗生物質を使用しないで済む。
- アデノウイルス感染症であることが分かれば，臨床経過の予測がしやすい

などが特記すべき点と考えられる。

1．迅速診断キット

アデノウイルス迅速診断法キットとして，免疫クロマトキットおよびELISAキットがある。

1）免疫クロマトキット（ICキット）

ろ紙中の標識抗体（金コロイド等，発色で確認できるもので標識）が，検体中のアデノウイルスと反応して，標識された抗体―抗原の複合物がろ紙中を毛細管現象で進む。毛細管現象で進む先に抗アデノウイルス抗体を結合させておくと，進んできた標識抗体―アデノウイルス複合体が補足されて目視確認できることによる手法である（図2）。1997年から市販され，2007年7月現在10種類のキットが市販されている（表1）。このうちチェックAd（アズウェル社），イムノカードSTアデノウイルス（マイアビー社）およびキャピリア®アデノ（日本ベクトン・ディッキンソン）は糞便検体と咽頭検体に使用できる。ラピッドテスタロタ・アデノ（第一化学薬品）と，ディップスティック®栄研アデノ（栄研化学）は糞便検査用である。キットは，適用検体が増えることがあり，この数年でも適用検体が増えたものが見られる。清水ら[3]によると，ラピッドテスタロタ・アデノは検出感度が$10^{6.0}$TCID50/mlで，同じ第一化学薬品から販売されているラテックス凝集反応キットより約10倍感度が良い。2007年7月現在，さらに喉頭用キット3種類および角結膜上皮細胞用キット1種類が市販されている（表1）。

免疫クロマト法の利点は，10～15分で結果が得られる迅速性と，操作の簡便性である。

2）ELISA

抗ヒトアデノウイルスモノクローナル抗体を利用した固相サンドイッチ法のELISAがキットとして市販されている（表1）。固相の抗体に結合したヒトアデノウイルスに酵素標識抗体が結合して，陽性の場合に基質と反応して発色することでヒトアデノウイルスの存在を確認できる手法である。キットの反応時間は標準法で70分，短縮法で40

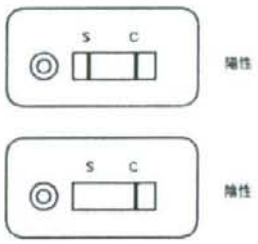

図2．免疫クロマト陽性例と陰性例の模式図

表1．ヒトアデノウイルス検出用　免疫クロマトおよびELISAキットの種類（2007年7月現在）

名　称	測定原理	発売元	使用検体	適用する感染症 呼吸器	眼	消化器	市販年
アデノクロン	ELISA	テイエフビー	糞便，角結膜上皮細胞，咽頭・扁桃上皮細胞	○	○	○	1992（呼吸器検体に適用拡大は2001）
アデノクロンE	ELISA	テイエフビー	糞便			○	1993
アデノチェック	IC*1	明治乳業	角結膜上皮細胞		○		1997
ディップスティック「栄研」アデノ	IC	栄研化学	糞便			○	2000
チェックAd	IC	アズウェル	咽頭結膜上皮細胞又は角結膜上皮細胞	○	○		2001
ラピッドテスタクト・アデノ	IC	第一化学薬品	糞便			○	2001
イムノカードSTアデノウイルス「眼・咽頭用」	IC	テイエフビー	角結膜上皮細胞，咽頭粘膜上皮細胞	○	○		2002
キャピリア　アデノ	IC	日本ベクトン・ディキンソン	角結膜上皮細胞，咽頭粘膜上皮細胞	○	○		2003
キャピリア　アデノ　アイ	IC	わかもと製薬	角結膜上皮細胞		○		2005
クイックチェイサー Adeno 咽頭	IC	ミズホメディー	咽頭粘膜上皮細胞	○			2006
ラピッドテスタ hs アデノ	IC	第一化学薬品	咽頭粘膜上皮細胞	○			2007
ホクテムSアデノ	IC	シスメックス	咽頭粘膜上皮細胞	○			2007

*1：免疫クロマト法

分である。ラテックス凝集反応や免疫クロマトキットと比較すると迅速性の点でやや劣る。しかし，表1に示した通り，1つのキット（アデノクロン：テイエフビー社）で呼吸器，眼および糞便のすべての測定が可能で，多検体を同時に測定できる利点がある。F種に属する40および41型はentericアデノウイルスと呼ばれ，感染症胃腸炎および乳幼児嘔吐下痢症の主要な病原体の一つである。ELISAキットのアデノクロンEは，F種を特異的に検出するので，下痢症患者の集団発生などでの病原体の特定に有用と考えられる。なお，F種は通常の細胞で難増殖性のウイルスである。

2．検査における注意点

ICキットによる検査は，検出感度がHeLa細胞によるウイルス分離と比較して80％程度であるので検査陰性の結果がアデノウイルス感染症を完全に否定するものではない。できる限り検出率を上げるためには次の2点が重要である。

・前述したとおり，検体採取においては採取時期が重要であるので発症4日以内の検体を用いる。

・検体採取で，炎症部位を強く擦過することが必要である。角結膜および咽頭・扁桃上皮細胞中にウイルスが存在するので，できる限り十分量の細胞を採取すること。

また，検査結果の評価においては，次の2点に注意する必要がある。

・便検体では，ICキットでは現在のところ，アデノウイルスが陽性か否かを調べているキットしかないため，C種アデノウイルスの持続感染を念頭に置くべきである。

・キットによって適用検体が異なるので，例えば咽頭検体用のキットで尿や糞便検体を試験した場合，偽陽性になる危険性があり，キットごとの適用検体と使用法を守る必要がある。

3．ICキットに関わる話題

ICキットの評価において，ウイルス分離法を基準とするのが，最も信頼のおける方法と考えられている。しかし，我々はICキットでアデノウイルスが検

出された検体で，ウイルス分離を実施するとコクサッキーウイルスB群が分離される例に遭遇した。この場合，ウイルス分離をゴールドスタンダードとするとICキットの検査結果が偽陽性と判定される。我々は，この検体がアデノウイルスとコクサッキーウイルスB群の重複感染によることをリアルタイムPCRで定量的に示した[5]。

ICキットの結果とウイルス分離の結果が異なる場合，重複感染の可能性を考慮すべきと思われる。

その他の診断法

迅速診断法以外のアデノウイルス診断法として，1．ウイルス分離，2．PCR検査，3．リアルタイムPCR検査，4．LAMP法，5．制限酵素切断法，6．ラテックス凝集法，7．電顕法，8．免疫蛍光法，が挙げられる。

1．ウイルス分離

培養した細胞内でウイルスを増やす手法である。培養細胞としてHeLa細胞（≒HEp-2細胞），RD細胞，A549細胞等を用いる。血清型によっては1ヶ月以上の期間を要するため迅速性がない点が大きな問題点である。特に8型などではウイルス分離に長期間を要する。しかし，生きたウイルスを得ることができ，現在でもゴールドスタンダードとされる。

2．PCR検査

数時間程度で結果が得られ，その後の塩基配列解析によって同一血清型内での系統解析等も可能であり，優れた検査法である。しかし，現在，アデノウイルス用PCRの系が多数存在し，その検出感度が異なるので，使用するPCR手法の選択が重要である。おおまかに，臨床検体中のアデノウイルスを直接検出できるものと，ウイルス分離後に適用するものがある。シークエンスおよび系統解析によって，血清型別が可能な場合が多い。我々は現在，アデノウイルスレファレンス活動を実施しており，多数存在するアデノウイルスに対するPCRの系を比較評価してマニュアル化する作業を実施している。

3．リアルタイムPCR検査

ウイルス遺伝子の定量が可能であり，迅速である点で有用性が高い。通常の系では，型別ができない点が欠点である。

4．LAMP法

日本で開発されたゲノム増幅・検出手法であり，若林ら[6]は眼検体から1型，3型，4型，8型，19型および37型を直接的に検出する手法を報告している。

5．制限酵素切断法

アデノウイルス40および41型は下痢症患者の糞便中に大量に排泄される。排泄されたウイルスから直接にDNAを制限酵素で切断し，その電気泳動パターンによりウイルスを同定する手法である。

6．ラテックス凝集法

アデノウイルスに対する抗体を結合させたラテックス粒子を用いて，アデノウイルスと結合して凝集塊が見られる場合に陽性と判定する手法である。ICキットより，やや感度が劣る。

7．電顕法

電子顕微鏡でアデノウイルス粒子を検出する手法である。現在でも下痢症検体で使用されている。

8．免疫蛍光法

気道，眼，尿，生検または剖検材料中の細胞内にアデノウイルス抗原が存在することを証明する方法として用いる。検査材料を低速遠心し，沈渣した細胞を固定してアデノウイルスに対する蛍光標識抗体でアデノウイルスを検出する。

これらの手法は，それぞれに特徴があり，ICキットおよびELISAの欠点である1．型別ができない点，2．定量ができない点，3．組織での局在を観察できない点，4．形態学的な観察ができない点，を補完する手法として使用することができる。

まとめ

近年，アデノウイルス迅速診断キットが小児科および眼科を中心とした臨床現場に普及しベッドサイドでのウイルス検査が可能となってきた。検査結果は，抗アデノウイルス薬が未だ存在しないため，特異的な治療には結びつかないが，二次感染を引き起こしやすいアデノウイルスを早期に診断する意義は感染拡大防止の点で大きい。免疫クロマトキットは簡便な操作で，高感度な実験室検査の80％程度の検出感度を持つが，検体採取時期および検体採取手技によって，検出率が下がることがあるので注意が必要である。検査陽性の場合は，特に糞便検体での結果の解釈に注意が必要である。検査陰性の場合は，アデノウイルス感染陰性と断定できない点に留意すべきである。

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Diagnoses for Adenoviral Infections

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Key Words Adenovirus, Immunochromatographic Kit, Diagnoses of Viruses, Rapid Diagnosis

短　報

白金—金コロイドイムノクロマトグラフ法を使用したアデノウイルス検査キットの有用性

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Key words: adenovirus, immunochromatography, rapid diagnosis

序　文

アデノウイルスは咽頭結膜熱などの呼吸器疾患，流行性角結膜炎などの眼疾患，また，感染性胃腸炎および出血性膀胱炎等，多彩な臨床症状を引き起こすウイルスである．現在，AからFの6種に分類され，51血清型が確認されている．近年，イムノクロマトグラフ法による検査キットがアデノウイルスの診断に用いられ，臨床現場において汎用されている．現在，市販されているアデノウイルス検査キットのほとんどが金コロイド標識抗体を使用している[1]．今回，新たに開発された白金—金コロイド標識抗体を用いたイムノクロマトグラフ法の検査キットであるイムノエース　アデノ（タウンズ）について，検出感度の基礎的検討を行ったので報告する．

材料と方法

川崎市で分離・同定されたAd1型～8型，11型，19型および37型の11血清型について，Karberの方法[2]によりTCID$_{50}$/mLを算定し，10段階（$\times10^{-1}$～$\times10^{-5}$）および2段階（×2，×4および×8）希釈したアデノウイルス希釈液を用いて最小検出感度を測定した．イムノエースアデノの比較対照として，金コロイド標識抗体を使用しているアデノウイルス検査キットであるキャピリアアデノ（日本ベクトンディッキンソン）およびチェックAd（アルフレッサファーマ）についても同様に最小検出感度測定した．最小検出感度を決めるにあたり2回以上の測定を行った．なお，各検査キットへのウイルス液の滴下量は100uLとし，滴下後15分後に判定した．

成績と考察

イムノエース　アデノは検査に供したすべてのアデノウイルスの血清型で反応し，白金—金コロイド標識抗体の特徴である黒色のラインが認められた（Fig. 1）．各検査キットの検出限界について，Table 1に示した．イムノエースアデノでは，検体中に1.4×10^{1}～1.8×10^{2} TCID$_{50}$/100uLのウイルス量があれば検出可能であった．その値は，血清型によって異なる（抗原エピトープの差，または目視判定誤差による2倍程度

Fig. 1　ImunoAce Adeno results

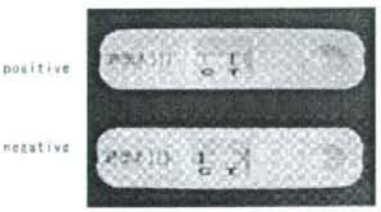

Table 1　ImunoAceAdeno vs. CapiliaAdeno vs. CheckAd.

Adenovirus type	ImunoAce Adeno	CapiliaAdeno	CheckAd.
1	8.0×10^{2}	3.2×10^{3}	1.6×10^{3}
2	8.0×10^{2}	3.2×10^{3}	3.2×10^{3}
3	1.4×10^{1}	5.6×10^{1}	5.6×10^{2}
4	5.0×10^{2}	1.0×10^{3}	1.0×10^{3}
5	1.6×10^{2}	3.2×10^{2}	3.2×10^{2}
6	1.6×10^{2}	3.2×10^{2}	3.2×10^{2}
7	5.6×10^{2}	1.1×10^{3}	1.1×10^{3}
8	2.5×10^{2}	1.0×10^{3}	1.0×10^{3}
11	1.8×10^{2}	9.0×10^{2}	9.0×10^{2}
19	8.0×10^{2}	3.2×10^{3}	3.2×10^{3}
37	8.0×10^{2}	3.2×10^{3}	3.2×10^{3}

(TCID$_{50}$/100uL)

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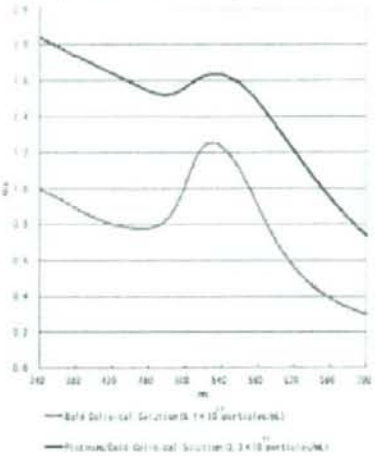
の訳者が見られたが，キャピリアアデノおよびチェックAdに比べて2～5倍検出感度が高かった．この検出感度の上昇がキットの添付文書に記載されたとおり，咽頭ぬぐい液で約10%検出率（57/52例）が既発品と比べ高い理由と考えられた．別のより感度が高い既発品と比較しても18%（57/56）感度が高い．交差反応性は，既発のキャピリアアデノ（タウンズ）と同等であった．後者のデータをより詳細に述べると182例の咽頭拭い液で陽性一致率98.1%（不一致例はPCRで陽性を確認），陰性一致率96.1%，全体一致率96.7%であった．

アデノウイルス感染症の検体採取部位は咽頭ぬぐい液あるいは結膜ぬぐい液が主で，検体採取者の手技によって検出感度に差が出る可能性がある．そのため，検査キットの検出感度は高いほうがよく，陽性ラインの色が強いほど，確実な判定が期待できる．今回検討したアデノウイルス検査キットのイムノエースアデノが使用している白金—金コロイドは従来の金コロイドに白金イオンをコーティングしたもので，黒色を呈している．白金—金コロイド溶液（3.3×10^{9}コロイド粒子/mL）および金コロイド溶液（9.1×10^{9}コロイド粒子/mL）について，分光光度計（日立U-2000）を使用し，波長340nm～700nmの範囲の波長をスキャンした場合，白金—金コロイドは全領域において金コロイドの吸光度を上回っていた（Fig. 2）．すなわち，白金—金コロイドは従来の金コロイドと比べて発色強度が高く，標識物としての視認性に優れているため，キットの感度も大幅に向上したと考えられる．また，色の違いだけでなく，白金—金コロイドの粒形が大きいことが検出感度に影響していると考えられる．Fig. 1に示したとおり，結果は明瞭であった．なお，Fig. 1の陽性で抗原量が多い検体であるため，抗原と結合したコロイド粒子がテストラインでトラップされ，コントロールラインがやや薄くなる従来と同じ現象が見られた．

本邦において，イムノクロマトグラフ法は各種感染症の検査キットとして広く使用されている．白金—金コロイド粒子を用いることは，現在市販されているイムノクロマトグラフ法の検査キットの検出感度を高める可能性が高く，今後，期待される手法であると考えられた．

Fig. 2. Colloidal Solution Absorption spectrum



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Evaluation of Immunochromatographic Detection Kit Using Adenovirus Pt-Au Colloid

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Short Communication

Detection and Quantification of Enterovirus 71 Genome from Cerebrospinal Fluid of an Encephalitis Patient by PCR Applications

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SUMMARY: Enterovirus 71 (EV71) is one of the causative agents of hand, foot, and mouth disease (HFMD) and is known to cause encephalitis, but several reports have identified EV71 in cerebrospinal fluid (CSF). We detected EV71 in CSF from a 20-month-old infant. The patient was diagnosed with brainstem encephalitis associated with HFMD. The clinical features of the patient were high fever (39.1°C) and myoclonic jerks, and magnetic resonance imaging of the brain showed a bright signal area around the 4th ventricle. From a nasopharyngeal swab and rectal swab, EV71 was detected using reverse transcription (RT)-nested polymerase chain reaction (PCR). From CSF, the EV71 genome was identified using pan-enterovirus RT-nested PCR and sequencing. By real-time PCR, the nasopharyngeal swab, rectal swab, and CSF contained 1.8×10^4, 9.8×10^4, and 1.8×10 copies of the EV71 genome/μL, respectively. The enterovirus could only be isolated by cell culture from the rectal swab, and it was identified by a neutralization test using EV71-specific antiserum. RT-nested PCR and real-time PCR are considered to be sensitive tools for EV71 diagnosis in CSF.

Enterovirus 71 (EV71) is one of the significant causative agents of encephalitis. In Japan, the first outbreak of hand, foot, and mouth disease (HFMD) associated with central nervous system (CNS) disorders occurred in 1973; the causative agent of the outbreak was EV71 (1). Large outbreaks of EV71 infection associated with CNS diseases occurred in Malaysia in 1997 (2) and Taiwan in 1998 (3). In 2000, the EV71 outbreak accompanied by CNS diseases occurred in Hyogo Prefecture, Japan (4). Usually, the detection and identification of EV71 from the cerebrospinal fluid (CSF) of encephalitis patients are known to be difficult (5). In this study we report an acute encephalitis case in which EV71 was detected in CSF using reverse transcription (RT)-nested polymerase chain reaction (PCR) and sequencing.

A 20-month-old girl was admitted to the pediatric ward of a hospital in Kakogawa city in Hyogo Prefecture with a high fever of 39.1°C, rashes on her hands, general weakness and poor feeding for a 2-day duration on July 1st of 2003. Encephalitis associated with HFMD was suspected based on clinical findings including vesicular rashes on the hands and feet, meningeal signs, somnolence, and myoclonic jerks. A magnetic resonance imaging (MRI) showed a high signal area around the 4th ventricle. Laboratory tests were in the normal range except for a leukocyte count of 13,900/μL, lactate dehydrogenase (LDH) of 278 IU/μL, and blood sugar of 175 mg/dL. CSF showed pleocytosis: 2,608/3 μL of cells; mononuclear cells 400 and multinuclear leukocytes 2,208.

The clinical specimens including CSF, pharyngeal swabs, rectal swabs, and sera were taken on admission under informed consent of the parents and sent to Hyogo Prefectural Institute of Public Health and Environmental Sciences.

Immediately after the arrival of the samples, viral RNA was extracted from the clinical samples using the QIAamp Viral RNA Mini Kit (QIAGEN, Heidelberg, Germany). The DNA fragment was amplified by RT-PCR using SuperScript™ II One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, Calif., USA) and Ex-*Taq* (TaKaRa, Shiga, Japan). For the detection of the enterovirus RNA in CSF samples, RT-PCR was performed using primers E2 (5'-CCTCCGGCCCCTGAATG) and E1 (5'-CACCGG ATGGCCAATCCA). Consequently, semi-nested PCR was performed using E2 and entR1 (5'-ATTGTCACCATAAGC AGCCA) to amplify a 154-bp fragment (4). The PCR was carried out using 94°C for 10 s, 60°C for 10 s and 72°C for 20 s, and was continued for 45 cycles; in the first cycle, the denaturing step continued for 5 min at 94°C, and in the last cycle, the extension step continued for 1 min at 72°C. The same PCR conditions were used for the first and second PCR. An aliquot (4 μL) template was used for the PCR. For the semi-nested (or nested) PCR, PCR products diluted 100 times (in water) were used.

For quantitative analyses, viral RNAs were extracted from a total of 140 μL of samples and eluted by 60 μL of the elution buffer. For cDNA synthesis, 15 μL of the 60 μL eluants were used. Thus, the original samples were calculated to include 1.71 times the number of copies of viral genome obtained by using real-time PCR. Therefore, the copy numbers in the samples for detection limit (copies/μL) experiments were multiplied by 1.71. The RT procedure prior to the PCR was performed using 45°C for 45 min and 94°C for 2 min. The temperature conditions were established using the DNA thermal cycler Dice (TaKaRa).

For amplification of the 5'-untranslated (5'-UTR)/VP4/VP2 region of enterovirus in the clinical samples, RT-PCR (6) was performed using P-2 (5'-CCTCCGGCCCCTGAATGCGGC

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TAAT) and E33 (5'-TCCGGGAATTTCAGTACCA) as the primers. Nested PCR was performed using the primer set EVP-4 (5'-CTACTTTGGGTGTCCGTGTT) and OL68-71R (5'-GGGAACTTCCAGTACCAYCC) (2). The PCR was carried out at 95°C for 10 s, 53°C for 45 s and 72°C for 45 s, and was continued for 40 cycles; in the first cycle, the denaturing step continued for 5 min at 95°C, and in the last cycle, the extension step continued for 4 min at 72°C.

Quantification of enterovirus was also performed by real-time RT-PCR (7,8). The primers and probes used were a forward primer (5'-TCCTCCGGCCCCTGA), reverse primer (5'-GATTGTCACCATAAGCAGCCA) and TaqMan Probe (5'-CGGAACCGACTACTTTGGGTGTCCGT). The machine used for the real-time PCR was an ABI PRISM 7700 (Applied Biosystems, Foster City, Calif., USA). In order to prepare a positive control standard for EV71, the RT-PCR product of an EV71 isolate (GenBank accession no. AB286954; position 1-190) was cloned into a plasmid using a TOPO TA cloning kit (Invitrogen). Plasmid DNA was purified using a HiSpeed Plasmid Midi kit (QIAGEN). The concentration of the plasmid DNA was measured by spectrophotometer at 260 nm, and the copy number/μL of inserted plasmid was calculated. Copies of EV71 genomes/μL were determined in clinical samples and serially diluted EV 71 genomes.

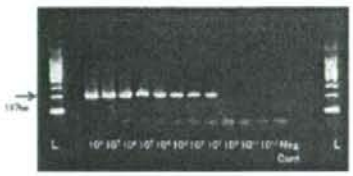

Fig. 1. Detection limit of PCR by primers E1 and E2. PCR result by primer E1 and E2 were shown. EV71 genome (1.9×10^6 copies/μL) were serially diluted to 10^6-10^{-1} copies/μL. Amplifications of PCR products (197 bp) were visible by an electrophoresis 10^6-10^1. The detection limit in original sample was calculated 1.7×10 copies/μL, because the (clinical) samples were 1.7 times diluted during genome extraction and cDNA synthesis procedure. L, 100-bp DNA ladder.

Table 1. Detection limits of PCR applications

PCR application	Calculated detection limit (EV71 genome copies/μL) in samples[a] ($n = 3$)
5'-UTR PCR	
primers E1+E2 (primer set 1)	1.7×10-1.7×10^5
primers entR1+E2 (primer set 2)	1.7×10^2-1.7×10^5
5'-UTR nested PCR[b] (primer set 1+2)	1.7-1.7×10
Real-time PCR	1.7-3.4×10^5

[a] Calculated from serially diluted cDNA of EV71 in this study.

By using serially diluted EV71 genome extracted from EV71 isolate in this study, the detection limits of the PCR applications were compared as shown in Fig. 1. The detection limit was calculated by using serially diluted cDNAs. Because the clinical sample size in this study was small, we deduced the detection limit of EV71 using repeated PCR diagnoses of these samples.

The detection limits of single PCR (primers E1 + E2) and semi-nested PCR (primers entR1 + E2) were 1.7×10-1.7×10^5 and 1.7-1.7×10 copies/μL, respectively. Single-PCR (primers entR1 + E2) had 1.7×10^2-1.7×10^5 copies/μL of detection limit for single-PCR use (Table 1). These results show that 5'-UTR nested RT-PCR was the most sensitive method. The real-time PCR method (detection limit, 1.7-3.4×10^5 copies/μL) was less sensitive than the 5'-UTR nested RT-PCR. The 5'-UTR nested RT-PCR was 10-10^5 times more sensitive to single 5'-UTR PCR applications. 5'-UTR/VP4/VP2 nested PCR could not detect EV71 in CSF and appeared to be less sensitive than other PCR applications in this report.

Enterovirus gene was detected from CSF, pharyngeal swabs, and rectal swabs using PCR. Serum samples were negative for enterovirus using all types of PCR applications in this study. From the real-time PCR, CSF, pharyngeal swabs, and rectal swabs contained enterovirus genome in 1.8×10, 1.8×10^4, and 9.8×10^4 copies/μL in clinical samples, respectively. The copy number 1.8×10 copies/μL obtained for CSF was within the detection limit of the real-time PCR application used in this study.

Virus isolation using GL37 cells (4) was positive only with rectal swabs, and EV71 was identified by a neutralization test using type-specific antiserum. No virus could be isolated from any specimens using RD cells. The results of PCR and virus isolation are summarized in Table 2.

EV71 was detected from CSF using RT-nested PCR and real-time PCR. The amount of EV71 virus in CSF was minute, approximately $1/10^6$ of the amount detected from rectal swabs. Using RT-nested PCR, cerebrospinal specimens were positive only in PCR for the 5'-UTR. Its sequence (AB183003) matched 100% with the appropriate region of the sequence of the EV71 strain isolated from rectal swabs (AB238695). These results indicated that it is difficult to detect EV71 from CSF because the amount of virus is very small. However, we detected EV71 using highly sensitive RT-nested PCR a day after hospital admission and were able to have the diagnosis results fed back to the clinical site. By using real-time RT-PCR, quantitative detection of the EV71 genome was possible. RT-nested PCR and real-time PCR are considered to be sensitive tools for EV71 diagnosis in CSF.

Detecting EV71 in CSF is significant because, as Koch's

Table 2. PCR and isolation results of EV71 from an encephalitis patient

Specimen	5'-UTR/VP4/VP2 nested PCR	5'-UTR nested RT-PCR	Cell culture using GL37 cells	real-time PCR (copies/μL)
Cerebrospinal fluid	-[c]	+[b]	-[c]	1.8×10
Pharyngeal swabs	+	ND[d]	-	1.8×10^4
Rectal swabs	+	ND	+	9.8×10^4
Serum	-	-	ND	ND

[b] EV71 positive.
[c] negative.
[d] not done.

postulates state, to detect a virus from a diseased organism is important; especially for enteroviruses, which are often isolated from healthy children. Detection of the EV71 genome from CNS samples can provide strong evidence of EV71 encephalitis. Yen et al. (9) reported that the detection limit of coxsakievirus B type 3 was 20 copies/μL. Their result was similar to our real-time PCR result.

The viral RNA extraction kit which was used in this study recommended concentrating samples of up to 3.5 mL to a final volume of 140 μL. This procedure was not included in this study. Further study of the effect of CSF concentration methods on CSF diagnosis is thought to be necessary.

Although the clinical sample number in this study is small, our results show that the PCR applications in this study are very sensitive and appear to be applicable to the diagnosis of EV71 in CSF.

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Short Communication

Molecular Epidemiology of Adenovirus Type 3 Detected from 1994 to 2006 in Hyogo Prefecture, Japan

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SUMMARY: The molecular epidemiology of 126 adenovirus type 3 (AdV3) isolates obtained in Hyogo Prefecture (population: 5.5 million) from 1994 to 2006 was studied. The hexon-coding region, including 7 hypervariable regions (HVRs) (1,419 bp), was sequenced. We found 5 nonsynonymous nucleotide substitutions in the HVRs. The results are strongly suggestive of positive Darwinian selection. We classified the AdV3 strains analyzed here into 3 genome types: AdV3x ($n = 44$), AdV3y ($n = 46$), and AdV3z ($n = 36$). AdV3x first appeared in 2001 in Hyogo Prefecture, and was detected predominantly during a large outbreak of AdV3 in 2003–2005. AdV3x was identical to a Korean strain responsible for a large outbreak of AdV3 in Korea in 1998–1999. We conclude that at least 3 genome types of AdV3 have circulated in Hyogo Prefecture, Japan, during the past 13 years (1994–2006). The findings also suggest that AdV3x was imported from Korea to Hyogo Prefecture in 2001.

Human adenoviruses (AdVs) are known to cause acute respiratory disease, pharyngoconjunctival fever, and epidemic keratoconjunctivitis (1-3). To date, a large family with 51 serotypes has been recognized. Among these serotypes, human adenovirus serotype 3 (AdV3) is the infectious agent most frequently isolated from patients with pharyngoconjunctival fever (PCF) (1).

National surveillance of PCF showed that the number of patients with PCF due to AdV3 increased in 2003 (4) and continued to increase until 2006. In Hyogo Prefecture (Fig. 1), which has a population of 5.5 million, an outbreak of PCF began in 2003 and continued until 2006 (5).

Recently, two new genome types, Ad3a16 and Ad3a18, were recognized during a large outbreak in 1998–1999 in Korea (6). Choi et al. have reported that new genome types of AdV3, isolated for the first time in 1998, are associated with 3 amino acid changes in the hexon-coding region, which potentially affects the antigenic characteristics of AdV3 (6). In this study, we report on the molecular epidemiology of AdV3 detected during the period extending from 1994 to 2006.

During 1994–2006, 3,711 clinical samples were tested as part of a surveillance program in Hyogo Prefecture. Among these clinical samples, 380 tested AdV3-positive by viral culture and polymerase chain reaction (PCR) analysis. From among these AdV3 samples, a total of 126 isolates (33%) were chosen for this study. At least 11% of the AdV3 samples per surveillance year were tested, except for in the year 1996, when no AdV3 was detected. The patients from whom these isolates were taken were diagnosed with PCF or another respiratory illness.

HeLa (HEp2), A549, RD, and Vero cells were used for the virus isolation procedure. Clinical samples were inoculated onto an 80% confluent cell monolayer in duplicate wells of a 24-well plate (Nippon Becton Dickinson, Tokyo, Japan). For cultivation, Dulbecco's modified Eagle's medium (Sigma, St. Louis, Mo., USA), supplemented with 2% heat-inactivated fetal calf serum and antibiotics, was used as the maintenance medium. The cells were passaged 3 to 8 times in order to allow time for the development of a cytopathic effect (CPE). When a CPE became evident, the isolates were tested by neutralizing antisera against AdVs purchased from Denkaseiken (Tokyo, Japan).

Fig. 1. Location of Hyogo Prefecture in Japan.

Viral DNAs were extracted using High Pure Viral Nucleic Acid Kits (Roche Applied Science, Basel, Switzerland). In the hexon-coding region, the hypervariable regions (HVRs) (7) were amplified and sequenced according to a modified version of the method reported by Takeuchi et al. (8); briefly, primers Hx5-NIID (5'-ATG GCT ACC CCT TCG ATG ATG CCC CAA T-3') and Hx3-NIID (5'-CTT ATG TGG TCG CGT TGC CGG CCG AGA ACG G-3') were used instead of HX5-1 and HX3-1. The underlined nucleotides were designed according to the AdV3 sequence in this study. Partial sequences of the hexon-coding region (1,419 bp), including the 7 HVRs

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(7), have been determined.

The sequences were aligned using GENETYX, version 6.1.2 (Software Development Co., Tokyo, Japan). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (version 4) developed by Tamura et al. (9). To construct a phylogeny tree, a neighbor-joining (NJ) method was used.

Among the 380 AdV3 isolates, 126 were sequenced and classified into 3 genome types. A comparison of the 1,419-bp hexon-coding regions revealed that there were 5 nucleotide replacements. All 5 nucleotide changes resulted in amino acid changes (Table 1). The 126 strains were analyzed and classified as follows: AdV3x ($n = 44$; 35%), AdV3y ($n = 46$; 37%), and AdV3z ($n = 36$; 29%).

Table 1. Sequence variation among adenovirus type 3 (AdV3) variants.

Genome type	Genome position corresponding to AY599836[a]				
	18915	19028	19663	19699	19730
AdV3x	C (Thr)[b]	G (Gly)	C (His)	G (Ala)	A (Asp)
AdV3y	A (Asn)	T (Val)	A (Asn)	A (Thr)	C (Ala)
AdV3z	C (Thr)	T (Val)	A (Asn)	A (Thr)	C (Ala)
Region	HVR 2[c]	HVR 3		HVR 7	

[a] AY599836 is a deposit number of DNA from AdV3 in USA.
[b] Amino acids shown in parentheses.
[c] Hypervariable region.

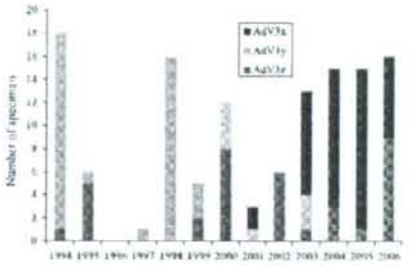

Fig. 2. Genetic classification of AdV3s detected in Hyogo Prefecture for 13 years (1994–2006).

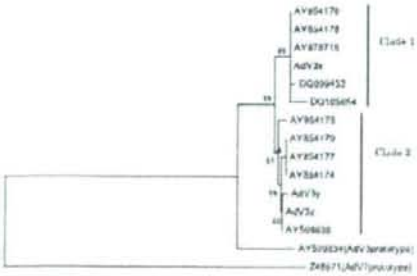

Fig. 3. Phylogenetic tree of the hexon-coding region generated by neighbor-joining method using 14 AdV3 strains and 1 AdV7 strain. The scale bar represents substitutions per site. The numbers at the nodes of the tree are bootstrap values in percentages, which measure the robustness of the support for each particular node.

AdV3x first appeared in Hyogo Prefecture in 2001 (Fig. 2), and it was identical to the Korean strain (Gen Bank accession no. AY854178). AdV3x was constantly dominant during 2003–2005. AdV3y was dominant in 1994 and 1998. AdV3y has not yet been deposited in GenBank. Although AdV3y is highly homologous to AdV3z, there is a single base substitution, accompanied by an amino acid change. AdV3z is identical to AdV3, which was isolated in the United States (AY599836), and detection of this variant was relatively continuous throughout the surveillance period of this study.

Phylogenetic analysis of AdV3x, AdV3y, and AdV3z was undertaken using 11 AdV3s and 1 AdV7 sequences available from GenBank. AdV3x belongs to Clade 1, which consists of recent East Asian isolates, and AdV3y and AdV3z belong to Clade 2 (Fig. 3).

Several other molecular epidemiology AdV3 studies in Japan have been previously reported, i.e., Guo et al. in 1988 (2), Mizuta et al. in 1994 (10), and Shiao et al. in 1996 (11). However, there is limited information from recent molecular epidemiological studies of AdV3 in Japan. Thus, a total of 126 AdV3 strains detected during 1994–2006 were analyzed and divided into 3 genetic types, using the hexon-coding region that includes 7 HVRs (7).

According to Crawford and Schnurr (7), unique sequences of AdVs are limited to the 7 HVRs, and 1 or more of these regions contain the type-specific neutralization epitopes. That study demonstrated that AdV neutralization epitope(s) are complex as well as conformational. Pichla-Gollon et al. (12) reported that the specific sites recognized by neutralizing antibodies have not been identified for any AdVs. They studied the major neutralization site for chimpanzee adenovirus 68, and found that a single small surface loop defines a major neutralization site for the AdV hexon.

AdV3x, first detected in 2001 in Hyogo Prefecture, is identical to the strain that caused a large AdV3 outbreak in Korea in 1998–1999 (6). Choi et al. has suggested that the genetic heterogeneity of AdV3 could play a potential role in the appearance of new genome types and could also affect the antigenic characteristics of AdV3. They also suggested a relationship between the appearance of a new AdV3 genome type and the large outbreak in Korea (6).

From the data available in the present study, it appears possible that AdV3x was imported from Korea to Japan and caused a large outbreak of AdV3 in Japan. A total of 2,368,877 people from Korea entered Japan, and a total of 2,319,676 Japanese people visited Korea in 2006, according to the Japanese Ministry of Justice.

All genome substitutions among AdV3x, AdV3y, and AdV3z were nonsynonymous, which is strongly suggestive of positive Darwinian selection. Additional studies will be necessary to provide detailed antigenic characterizations of AdV3x, AdV3y, and AdV3z. We conclude that at least 3 genome types of AdV3 circulated in Hyogo Prefecture, Japan, during the past 13 years (1994–2006).

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短　報

エンテロウイルス遺伝子診断法における市販RNA抽出キット選択の影響

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Key words: enterovirus, RNA extraction kit, extraction efficiency

序　文

近年，病因ウイルスの究明には遺伝子診断法が普及し，エンテロウイルスにおいても広く実施されている．遺伝子診断法における要となるのが，エンテロウイルスRNAの抽出操作である．1990年代後半からウイルスRNA抽出キットが数種類市販されているが，市販抽出キットの選択が結果に関与する可能性に関しては，ほとんど報告が見られない．そこで本研究では，臨床検体を材料とし，市販される3種類のRNA抽出キットを用いて，標準法[1]に準じたRT-PCRおよびSemi-nested PCRによるエンテロウイルス検出を試み，その結果を比較検討した．

方　法

1. 臨床検体

臨床検体には1994年8月から2005年11月までに兵庫県内で，感染症発生動向調査の一環として臨床症状からエンテロウイルス感染症が強く疑われた21名から採取した咽頭ぬぐい液を使用した．

2. エンテロウイルス分離株

エコーウイルス9型の標準株(Hill株)を，10^{5}TCID 50/mLより10^{-2}TCID 50/mLまでの力価に調整し使用した．

3. RNA抽出

市販のRNA抽出キットよりフィルター付きカラムを使用するタイプ，すなわち，QIAamp Viral RNA Mini Kit(キットA)(QIAGEN)，E. Z. N. A Viral RNA Isolation Kit(キットB)(Nanonex Technology Pte Ltd)，High Pure Viral RNA Kit(キットC)(Roche)を選択した．操作はキットの添付書に従い，インキュベーションや遠心操作等に選択の幅がある場合は，抽出されるRNA量がより多くなる方法を採用した．分離株は，各希釈あたり6回の抽出操作をくりかえし，キットの抽出効率の定量化に供した．

4. 遺伝子増幅

RT-PCRおよびSemi-nested PCRによるエンテロウイルス遺伝子の増幅を行い，RNA抽出の良否を判断した．操作による影響を排除するため，抽出操作がすべて終了した後に，全検体を同時にRT-PCR以降の操作に供した．RT-PCRには1ステップ，1チューブで操作が完了するキットである，Access RT-PCR System(Promega)を使用した．Semi-nested PCRにはEx Taq(TaKaRa)を使用した．プライマーはRT-PCRには上流側に60-80F：5'-AAA TCC TTG AKC GCC TGT TTT A-3'を，下流側にOL68-71R：5'-GGG AAC TTC CAG TAC CAY CC-3'を，Semi-nested PCRには上流側にEVP4：5'-CTA CTT TGG GTG TCC GTG TT-3'を，下流側にOL68-71Rを使用した．各反応条件は標準法[1]に従った．

PCR産物を1.5%アガロースゲルによる電気泳動の後，エチジウムブロマイドで染色し，紫外線下で増幅バンドの有無を確認した．

5. 統計学的解析

統計学的解析にはフィッシャーの正確確率検定を用いた．統計学的有意差は$p<0.05$として定義した．

結　果

1. 臨床検体を用いたエンテロウイルス検出

臨床検体からのエンテロウイルス検出率はRT-PCRでは，キットAで24%(5/21)，キットBで43%(9/

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Table 1 Clinical samples from 21 patients with aseptic viral meningitis infection, with a comparison of RT-PCR and semi-nested PCR results

Sample No.	Serotype of isolated virus	Sample collection date	Gender	Age	Clinical Diagnosis	DDBJ Blast	Accession Number of DDBJ	RT-PCR and semi-nested PCR results					
								Kit A		Kit B		Kit C	
								1st	2nd	1st	2nd	1st	2nd
1	EV71	1994/8/16	M	1Y5M	HFMD	EV71	AB252911		-		+		+
2	CA16	1994/9/9	M	8Y	HFMD	CA16	AB252910		+		+	+	+
3	ECHO9	1994/9/16	M	11M	erupsion	ECHO9	AB252913	+	+	+	+	+	+
4	EV71	1994/10/17	M	1Y11M	HFMD	EV71	AB252919		+	+	+		+
5	Polio 1	1994/11/11	F	7M	herpangina	Polio 1	AB252921		+	+	+	+	+
6	EV71	1995/11/5	M	2Y	HFMD	EV71	AB252905						
7	EV71	1995/12/12	F	1Y	aseptic meningitis	EV71	AB252901				+		
8	EV71	2000/9/8	F	1Y5M	HFMD	ND							
9	EV71	2004/7/11	M	2Y	herpangina	CA10	AB262508	+	+	+	+	+	+
10	EV71	2003/7/26	F	1Y	HFMD	ND							
11	EV71	2003/7/27	F	0Y3M	aseptic meningitis	EV71	AB285413		+		+	+	+
12	CA16	2004/8/21	M	1Y	HFMD	ND			+	+	+		+
13	CA4	2004/8/5	F	1Y	aseptic meningitis	CA4	AB262504	+	+	+	+	+	+
14	CA2	2004/8/16	M	2Y	herpangina	CA2	AB262500	+	+	+	+	+	+
15	CA9	2004/8/19	F	1Y	herpangina	CA9	AB262507		+		+		+
16	CA2	2004/9/11	M	2Y	herpangina	CA2	AB262501		+		+	+	+
17	CA6	2005/6/13	M	2Y	herpangina	CA6	AB262503	+	+	+	+	+	+
18	NT	2005/6/20	F	1Y4M	herpangina	Rhino	AB262925		+		+		+
19	CA6	2005/6/30	M	8Y	herpangina	CA6	AB285906		+		+		+
20	NT	2005/10/21	M	4Y	herpangina	ND							
21	CA10	2005/11/9	F	3Y	herpangina	CA10	AB262509		+	+	+	+	+

EV: enterovirus, ECHO: echovirus, CA: coxsackievirus A, HFMD: hand foot and mouth disease
DDBJ Blast: Results of DDBJ DNA Data Bank of Japan Blast (Basic Local Alignment Search Tools)
NT: enterovirus not typed, ND: not done

21)，キットCで48%（10/21）となった．一方，Semi-nested PCRでは，キットA およびCで76%（16/21），キットBで81%（17/21）となった．使用する抽出キットにより異なった検査結果となった事例が確認された（Table 1）．

2．分離株を用いたエンテロウイルス検出

ウイルス量が10^{3}TCID 50/mL以上で各キットともRT-PCR陽性となった．10^{2}TCID 50/mLの希釈系においてはキットAで17%（1/6），キットBで83%（5/6），キットCで100%（6/6）の検出率であり，フィッシャーの正確確率検定により$p=0.012$で有意差が認められた．キット間ごとの統計学的有意差はキットAとキットCの10^{2}TCID 50/mLの希釈系で$p=0.015$で認められた．10^{1}TCID 50/mL以下の希釈倍率ではすべてのキットで陰性となった．

Semi-nested PCRでは，検出限界が10^{-1}TCID 50/mLであり，統計学的有意差は認められなかった．

考　察

本研究において臨床検体に直接適用したRT-PCRの結果が，使用したRNA抽出キットにより異なる事例が確認された．我々はエンテロウイルス遺伝子診断法に際し臨床検体を用いる場合，偽陰性となる事がある危険性を指摘していた[3]．本研究により，その要因の一つとして，実験に用いたRNA抽出キットが関与する事例が確認された．

樽島ら[5]はヘルパンギーナ患者36名の臨床検体について，トリゾール（Invitrogen）を用いたRNAの抽出の後，RT-nested PCRを行い，乳のみマウスと同じ検出結果であったことを報告している．Knepp[6]らは，ポリオ2型の分離株から，自動核酸抽出機械，マニュアル抽出キット（キットA）およびトリゾールを用いてRNAの抽出を行い比較しているが，検体中のウイルス量が多い場合ではどの方法も同様の検出率となるが，臨床検体では結果が必ずしも一致していなく，我々の結果と一致する．しかし，彼らの研究はマニュアル抽出キット間の検出効率を検討したものでなく，我々はマニュアル抽出キット間の差を比較検討した．

臨床検体のRT-PCRの結果にキット間で差が見られた要因は，ウイルス希釈実験の結果から，臨床検体中のウイルス量が少ない場合に，各キットの抽出効率の差が顕在化したものと考えられた．なお，Semi-nested PCRを行うと，各キット間に有意な差はみられなくなった．

臨床診断上，ウイルス検出結果は極めて重要で，抽出キットの選択によりその成績が左右される可能性を

示唆された．今日，遺伝子解析による試験検査には市販のRNA抽出キットの利用がその簡便性から不可欠であるが，適用するウイルスや実験系により今回とは異なる実験結果となる可能性も考慮し，実験系ごとに用いる各キットの有効性を確認しておくことが必要と考えられた．

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Influence of Genome Extraction Kits on Enterovirus Detection Using RT-PCR

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ORIGINAL ARTICLE

Detection of Dual-Infected Cases of Adenoviruses and Coxsackieviruses Type B by Real-Time PCR but not by the Conventional Viral Culture Technique

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SUMMMARY

The aim of this study was to evaluate the applicability of diagnostic methods for dual-infected cases of human adenoviruses (AdVs) and coxsackieviruses type B (CBs). For this purpose, 100 nasopharyngeal samples from patients with acute exudative tonsillitis and clinically suspected AdV infection were analyzed. Using PCR and real-time PCR techniques for AdVs and CBs, we found 86 AdVs-only positive samples; we also found five dual-infected samples containing 5.4×10^5 to 7.1×10^8 copies/mL of AdV genomes and 1.4×10^4 to 1.3×10^5 copies/mL of CB genomes. By viral culture using A549 cells, two co-infected samples, which contained over 10^8 copies/mL of AdV genomes and $<10^5$ copies/mL of CB genomes, became AdV dominant, while three samples with less than 2.0×10^6 copies/mL of AdV genomes became CB dominant. An immunochromatography kit for diagnosing AdVs at the bedside was positive for 3/5 dual-infected patients, and PCR techniques for AdVs and CBs were both positive for 5/5. Viral culture is usually considered to be the gold standard for AdV diagnosis, but our results demonstrate the importance of PCR applications for the detection of AdV and CB genomes, particularly in clinical cases of suspected AdV infection. Even though the sample size of dual infection (n=5) is small, our results show the existence of dual infection cases which were difficult to diagnose by viral culture alone. (Clin. Lab 2007;53:XXX-XXX)

KEY WORDS

Dual infection, viral culture, adenovirus, coxsackievirus type B

INTRODUCTION

Even though plural diagnostic tools are currently available for human adenoviruses (AdVs) and enterovirus infections, the viral culture technique still remains the gold standard for the diagnosis of AdVs and coxsackieviruses type B (CBs) because serotype is an important biological phenotype closely related to immunity and, in some cases, to clinical presentation. AdVs and CBs replicate and produce cytopathic effects (CPE) in continuous human cell lines of epithelial origin such as HeLa (1), except for AdV types 40 and 41. The nature and speed of the CPE are characteristic of the particular virus involved. Therefore CPE represents an important criterion for the preliminary identification of clinical isolates. However, the CPEs of AdVs and CBs are sometimes similar, especially when viral load is high.

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AdVs and CBs have a similar target organ, utilize the same virus receptor (2), and share cell sensitivity and pathogenicity, but the biochemical features of AdVs and CBs are quite different. For example, AdVs are double-stranded DNA viruses and CBs are single-stranded RNA viruses. Therefore, genetical and immunological discriminations of AdVs and CBs are usually easy. However, we have found that diagnosis of dual infection with these viruses is not easy using viral culture alone.

After real-time PCR became available for diagnosis of AdVs (3) and enteroviruses (EV) (4), direct quantitative detection of these viruses in clinical samples was a possibility. Additionally, immunochromatographic-kit (IC-kit) tests for AdV infection became available in the 1990s. We have found that the results of these newly developed methods sometimes disagree with viral culture results. When only CB is isolated by IC-kit from an AdV-positive sample, it could be considered a false positive because viral culture is usually considered the gold standard. Recently, such conflicting results have become increasingly common.

In this study, we evaluated five dual-infected cases of AdVs and CBs qualitatively and quantitatively by viral culture, IC-kit, PCR and real-time PCR applications. In this report, we use the term "dual infection" to refer to co-infection with AdVs and CBs for simplicity of terminology.

MATERIALS AND METHODS

Patients

From January through June 2003 outpatients suffering from acute exudative tonsillitis (n=100) at a clinic in Himeji city (460,000 people), located in the central area of Japan, were enrolled in the study. The mean age of the patients was 4.1 ± 2.1 years (mean ± standard deviation [SD]). The sample collection protocol was reviewed and approved by the local institutional review board, and informed consent was obtained from all patients (patients' parents or guardians).

Clinical samples

Nasopharyngeal swabs (n=100) from the patients with acute exudative tonsillitis were used. The swab sample was extracted with 500 µL of phosphate-buffered saline and an aliquot of 200 µL was used for IC-kit analysis with Check Ad (AZWELL, Osaka, Japan) at the bedside. The remaining approximately 200 µL portion was transferred into a tube containing 2 mL of Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) and used for viral culture, PCR and real-time PCR applications.

Viral culture and identification

Clinical samples were inoculated onto an 80% confluent monolayer of A549 (obtained from the JCRB cell bank, Osaka, Japan) or HeLa (obtained from the National Institute of Infectious Diseases, Tokyo, Japan) cells in duplicate wells of a 24-well plate (Nippon Beeton Dickinson, Tokyo, Japan). A549 cells were used for dual-infected samples. For cultivation, Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 2% heat-inactivated fetal calf serum and antibiotics was used as a maintenance medium. The cells were passaged three to eight times to allow time for CPE to develop. When the CPE became evident, the isolates were tested by a neutralization test using antisera against CBs 1-6 and AdVs 1-7 purchased from Denkaseiken (Tokyo, Japan).

Viral genome preparation

AdV DNAs and EV RNAs were prepared directly from a total of 140 µL of nasopharyngeal sample or viral isolate by using a Qiagen Viral RNA kit (Qiagen, Hilden, Germany). Nucleic acid was eluted in 50 µL of supplied elution buffer and stored at -80 °C until use.

Direct detection and identification of AdVs in clinical samples

The extracted AdV DNAs were detected and identified by a multiplex PCR protocol (5), nested PCR and sequence analysis (6). IC-kit was used at the bedside of the patients as described previously (7).

Detection and identification of CBs in clinical samples

All CBs in clinical samples could be isolated using HeLa cells. These isolates were identified by neutralization tests. Therefore, direct detection and identification of CBs were technically not necessary. However, we confirmed CBs in clinical samples by RT-PCR-based sequencing using VP4 regions (8).

Real-time PCR

To determine viral genome copy number/mL, real-time PCR applications for AdVs (3) and EVs (4) were used. The machine used for the reactions was an ABI PRISM 7700 or ABI PRISM 7900 HT (Applied Biosystems, Foster, CA, USA). Copy number/mL was determined in clinical samples and isolates. In order to prepare a positive control standard for EVs, the RT-PCR product of an enterovirus 71 isolate (GenBank accession No. AB286954) was cloned into a plasmid using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was purified using a HiSpeed Plasmid Midi kit (Qiagen Hilden, Germany). The concentration of the plasmid DNA was measured by spectrophotometer at 260 nm and the copy number/mL of inserted plasmid was calculated.

Table 1. Virus Detection and Identification in Throat Samples of Acute Exudative Tonsillitis Patients (n=100)

PCR and sequencing	IC-kit for AdV		Viral culture in HeLa or A549 cells	
	positive	negative	positive	negative
AdV1	8	2	10	
AdV2	14		14	
AdV3	55	3[b]	56	2[b]
AdV5	2		2	
AdV6	2		2	
CB2		1	1	
AdV3+5	1		1	
AdV3+CB2	2	1	3	
AdV3+CB5	1		1	
AdV1+CB2		1	1	
AdV NT[a]		3		3
negative		4		4
Total	85	15	91	9

[a]NT indicates not typed

RESULTS

Patients

All acute exudative tonsillitis patients in this study were suffering from a fever of 38.0 - 40.7 °C (39.4 ± 0.6: mean ± SD). Five dual-infected patients were clinically indistinguishable from the other AdV-only cases. The age of the dual-infected patients ranged from 2 to 5 years (4.1 ± 1.4: mean ± SD). They suffered from a high fever of 39.5-39.7 °C (39.6 ± 0.1: mean ± SD). Of these five cases, two were sporadic and three were epidemic. These five cases occurred in different periods of time and a different combination of AdVs and CBs serotypes was detected in the three epidemic cases. Therefore, these cases were assumed to have no direct relationship to one another.

Detection and typing of viruses

Of 100 patients with acute exudative tonsillitis, viruses were detected in 96 patients and identified as shown in Table 1. Of these 96 patients, 95 were AdV-positive by PCR except for one CB2-isolated patient. A total of 91 patients were culture-positive by HeLa or A549 cell culture. IC-kit results at the bedside were positive for 85 patients. Five dual-infected cases and one co-infection with AdV3 and AdV5 were detected by PCR and sequencing (5, 6).

Quantitative analysis of dual-infected samples

Five dual-infected samples were quantified by real-time PCR methods for AdVs (3, 7) and EVs (4). As shown in Table 2, clinical samples included 1.5×10^{6} - 4.0×10^{9} copies/mL of AdV genomes and 1.4×10^{4} - 1.3×10^{9} copies/mL of CB genomes. IC-kit-negative samples (n=2) contained 1.5×10^{5} - 4.2×10^{5} copies/mL of AdV genomes and positive samples (n=3) contained 5.6×10^{6} - 4.0×10^{9} copies/mL of AdV genomes. IC-kit-positive samples contained a higher copy number of AdV genomes than negative samples.

Real-time PCR analysis of AdVs after viral culture in HeLa cells

Two dual-infected samples which contained 5.1×10^{3} times and 1.7×10^{3} times the copy number of AdVs relative to CBs (Table 2, Nos. 1 and 3, respectively) were positive by real-time PCR for AdVs after viral culture in HeLa cells. However, the other three samples, which contained 4.2×10^{4}, 1.7×10^{2} and 2.9×10^{3} times the copy number of AdVs in comparison to CBs (Table 2, Nos. 2, 4 and 5 respectively), were below the detection level for AdVs of the same real-time PCR protocol after viral culture in HeLa cells. The real-time PCR-positive culture samples contained 5.3×10^{9} and 2.3×10^{8} copies/mL of the AdV genome, respectively. No propagation of AdV was observed with these two samples after viral culture in HeLa cells.

Viral culture using A549 cells

The five dual-infected samples described above were inoculated onto A549 cells, and the two samples which contained over 10^{6} copies/mL of AdV and $<10^{5}$ copies/mL of CB became AdV dominant. These samples were identified as AdV 3 using neutralization tests.

Real-time PCR analysis of CBs after viral culture in A549 cells

Three dual-infected samples which contained 1.7×10^{2} - 4.2×10^{4} times the copy number of CBs in comparison to

Table 2: Results of Viral Cultures, PCR, Real-time PCR and IC-kit Analysis of Dual-infected Clinical Samples from Acute Exudative Tonsillitis Patients (n=5)

Patients			Viral culture		PCR and sequencing		Copy number by real-time PCR (clinical sample direct)			IC-kit for AdV at the bedside
No.	Age	Sex	HeLa cells	A549 cells	PCR	RT-PCR	AdV	CB	AdV/CB ratio	
1[a]	3y 2m	female	CB2	AdV3	AdV3	CB2	7.1×10^{8}	1.4×10^{4}	5.1×10^{4}	+
2	2y 4m	female	CB2	CB2	AdV3	CB2	5.4×10^{5}	1.3×10^{9}	4.2×10^{-4}	-
3[a]	5y 4m	female	CB5	AdV3	AdV3	CB5	1.4×10^{8}	8.2×10^{4}	1.7×10^{3}	+
4	4y 5m	male	CB2	CB2	AdV3	CB2	2.0×10^{6}	1.2×10^{8}	1.7×10^{-2}	-
5	5y 5m	female	CB2	CB2	AdV1	CB2	1.5×10^{6}	5.1×10^{8}	2.9×10^{-3}	-

a) two patients from whom a different virus was isolated by HeLa cells and A549 cells

AdVs (Table 2, Nos. 2, 4 and 5) were positive by real-time PCR for CBs after viral culture in A549 cells. However, two samples, which contained over 10^6 copies/mL of AdV and $<10^3$ copies/mL of CB in the clinical samples, fell below the detection level of the same real-time PCR protocol for CBs. These results were concordant with the neutralization results described above.

Real-time PCR analysis of CBs after viral culture in HeLa cells

Two samples fell below the detection level after culture in A549 cells. These two samples contained 2.6×10^6 and 3.8×10^9 copies/mL of CBs after viral culture in HeLa cells. About 10^3 propagations were observed in HeLa cells.

DISCUSSION

Although viral culture is the gold standard for AdV and enterovirus diagnosis, our results show that the results obtained by culture technique alone are sometimes misleading when a sample contains both of these viruses. All of these cases were identified as CB infection alone by viral culture using HeLa cells. Because in these cases no significant propagation of AdVs was observed in HeLa cells, dual infections of CBs and AdVs were difficult to identify. When 10^3-fold or greater AdVs relative to CBs were present in a sample, AdVs became dominant using A549 cells. Two out of five dual-infected samples belonged to this category. After isolation from HeLa cells, these two samples were positive by real-time PCR. However, no propagation was observed.

CBs are known to use the same cell receptor as AdVs (9). However, species B AdVs do not use the coxsackie-adenovirus receptor (CAR) as a cellular fiber receptor (2). Our co-infected cases, except for one case, included AdV 3 which belongs to species B. Therefore, CAR could not explain the poor propagation of AdV 3. It is possible that the presence of enteroviruses interfered with the replication of AdVs in tissue culture. Lee et al. (10) compared A549 and BGMK cells for isolation of AdVs from an aquatic environment and reported that they obtained a higher viral frequency with A549 cells than with BGMK cells. They deduced that the adenoviral frequency obtained with BGMK cells could be influenced by interference from enteroviruses, and thus AdVs in aquatic environments could be very insufficiently propagated in BGMK cells. Poor propagation of AdVs using HeLa cells might be explained in the same manner. We compared the cell sensitivity of A549 and HeLa cells using AdV isolates and found that A549 cells had an approximately 10-fold higher TCID50 titer (data not shown). Woods and Young (11) compared A549 and other cell lines and reported that A549 cells were optimal for recovery of AdVs from clinical specimens. The A549 cell line was also reported to be suitable for recovering a wide range of AdVs and enteroviruses (10). In this study, we confirmed the usefulness of A549 for recovering AdVs from CB co-infected clinical samples.

Previously we (7) reported that viral culture-negative and PCR-positive samples contained 3.0×10^4 to 3.8×10^5 copies of the AdV genome/mL. In this study, three dual-infected samples which contained 5.4×10^5 to 2.0×10^6 copies of the AdV genome/mL became AdV culture-negative even by HeLa and A549 cells. To avoid interference with co-infected CBs, we tried isolation of AdVs after neutralization of CBs in clinical samples. However, no AdV was isolated from these samples (data not shown). We also tried to isolate AdVs exclusively using RD cells, which are usually described as having no sensitivity to CBs (12), and the result was negative (data not shown). These results suggest that these three samples contained a concentration of AdVs that was under the isolation detection level.

In this study we found two dual-infected samples which contained a much higher load of AdVs in comparison to CBs. As described above, only CBs were isolated by HeLa cells. But using A549 cells, only AdVs were isolated. It seemed that the ratio of AdV to CB explains these differences. To explain these results, further examinations using higher sample numbers are needed.

In a previous report (7) we were able to isolate virus from all IC-kit-positive samples. But in this study, we were not able to isolate virus from one sample that was IC-kit-positive at the bedside. By real-time PCR it contained 2.0×10^{6} copies/mL of the AdV3 genome. Freeze and thaw cycles which were unavoidable during the period of this study may have damaged the AdV particles. This effect might explain the culture-negative result.

Although infections with AdVs and CBs mostly remain asymptomatic, both AdVs (13) and CBs (14) sometimes cause fatal infections and it is important to diagnose these viruses correctly. Correct laboratory diagnosis has an impact on proper infection control. If viral culture is considered to be the gold standard for AdV diagnosis, AdV-positive results obtained by IC-kit or PCR applications could erroneously be regarded as false positives. There was no clinical difference between dual-infected patients and other AdV-infected patients. Therefore, AdV seemed to be the main causative agent of acute exudative tonsillitis in these patients. Moreover, because we found five clinical samples in which it was difficult to isolate both AdVs and CBs by viral culture from dual-infected samples, PCR applications seemed important when CBs are isolated from patients with clinically suspected AdV infections.

The sensitivity of both techniques, i. e. virus isolation in tissue culture and PCR depends on amplification of the virus or its nucleic acid. However, in the case of double infections, amplification of one of the two viruses in culture seems to be inhibited by the other through so far undefined interference mechanisms. In contrast, amplification of nucleic acid in PCR works in parallel and without significant interference, leading to the detection of the major and the minor component of double infections, thus demonstrating the true epidemiological situation.

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