②新型インフルエンザワクチン優先順位

CATEGORY 1
- 呼吸器系の基礎疾患をもつ児（気管支喘息を含む）〔理由：当院入院例の約半数に気管支喘息の既往の児が含まれている〕
- 神経筋疾患をもつ児〔理由：海外の報告で，小児の死亡例の基礎疾患の9割を占める〕
- 血行動態に影響のある心疾患をもつ児

CATEGORY 2
- 妊婦〔理由：海外の報告で，入院例，重症化例が多い〕

CATEGORY 3
- 免疫不全患者（先天性，後天性，化学療法後など）
- 内分泌疾患をもつ児（糖尿病など）
- 肝臓疾患をもつ児（肝不全など）

（当院の疾患別優先順位）

※13
http://www.mhlw.go.jp/stf/shingi/2r9852000000s2wz-att/2r9852000000s307.pdf

患者に対するワクチン接種

2009年12月になり，患者へのワクチン接種が可能となり，患者にも同様に優先順位をつけ（②），新型インフルエンザワクチン外来を設け，接種を開始した．

とくに感染すると重症となると推定されていた免疫不全，呼吸器疾患，循環器疾患患者，妊婦には，優先的に接種を行ったが，その初期には接種数に限りがあり，数時間でワクチンがなくなり，接種希望者に十分なワクチンが行き渡らない状況が続いた．しかしながら，近医での接種が可能となり，また2010年1月になると大量のワクチンが接種可能となり，流行のピークが過ぎていったことも相まって，新型インフルエンザワクチン外来への患者は激減し，2010年1月末には閉鎖した．

今後の課題

厚生労働省の新型インフルエンザ対策総括会議の報告書から

今後の課題に関しては，厚生労働省の新型インフルエンザ対策総括会議の報告書が発表されており[※13]，この報告書の中での全般的事項として，次の5点が指摘されている．

① 病原性等に応じた柔軟な対応（感染力だけでなく致死率などを総合的に考慮したうえでの対策の選択）
② 迅速・合理的な意思決定システム（国における意思決定プロセスと責任主体を明確化するとともに，医療現場や地方自治体などの現場の実情や専門家の意見を的確に把握し，迅速かつ合理的に意思決定のでき，かつ透明性のあるシステムづくり）
③ 地方との関係と事前準備（関係者間で対処方針の検討や実践的な訓練を重ねるなどの準備，また，地方の役割分担の事前の確認が必要）
④ 感染症危機管理に関わる体制の強化（国，地方における感染症対策に関わる危機管理を専門に担う組織や人員体制の大幅な強化，人材の育成，関係機関のあり方や相互の役割分担，関係の明確化等が必要）
⑤ 法整備（感染症法や予防接種法などを含めた，感染症対策全般のあり方の見直しなど）

一方，各論として，①サーベイランスの充実，②広報，リスクコミュニケーションの改善，③水際対策のあり方の再検討（空港での検疫をどこまで行うか），④公衆衛生対策（学級，学校閉鎖のあり方など），⑤医療体制のあり方（発熱外来，発熱相談センターなどの存在意義），⑥ワクチン制度の見直し，運用上の問題点などがあげられている．

抗インフルエンザ薬の適正使用

日本での新型インフルエンザ感染症による死亡率は100万人あたり0.2人であり，他の国々と比較しても非常に低い率であった（米国3.3，英国2.2，オーストラリア8.6）[6]．今回の新型インフルエンザ感染症では，国内で多

- 今後流行が懸念される高病原性鳥インフルエンザ（H5N1），あるいは他のインフルエンザ感染症のアウトブレイクに対しては，今回の新型インフルエンザ対応の教訓を生かした対応が必要である．
- 貴重な医療資源である抗インフルエンザ薬の適正使用について，今後検討が必要である．
- 今後流行が懸念されるインフルエンザ感染症に対するワクチン接種に関しては，その政策を国策として考える専門委員会の設置が必要である．

くの患者に抗インフルエンザ薬が処方されたが，それが死亡率の低かった一つの理由となっていることは確かであろう．抗インフルエンザ薬を発症後48時間以内に投与すると発熱期間が1.5～2日短縮することが知られている[7)・※14]が，インフルエンザは基本的に自然治癒する疾患である．

今回は，季節性インフルエンザとは別の過去に経験のないインフルエンザ感染症であるということで，抗インフルエンザ薬が多用された．限られた医療資源を有効に使うためにも，また今後起こりうる抗インフルエンザ薬に対する耐性獲得も考慮して，日本での抗インフルエンザ薬投与の効果について再度検討する余地があると思われる．

※14
インフルエンザの合併症の予防を支持するデータは存在しない．

予防接種政策を検討する専門委員会の必要性

ワクチン接種においては，米国では8月末にすでにACIPが新型インフルエンザのワクチンの供給体制，接種対象者などを発表していたのに対して[8)]，日本国内では，その対応で混乱していた．このような臨時の体制に対応するためにも，常時ワクチン政策を国策として検討する専門委員会を常設し，継続的に方針を立てていかなくてはいけない[※15]．

ACIP：Advisory Committee on Immunization Practice

※15
わが国にも，インフルエンザワクチンのみならず他のワクチンに対してもACIPのような専門家の政策集団が必要で，今後その設立と積極的な活動が望まれる．

文献

1) Outbreak of swine-origin influenza A (H1N1) virus infection : Mexico, March-April 2009. MMWR Morb Mortal Wkly Rep 2009 ; 58 : 467-70.
2) Up to date : swine influenza A (H1N1) infections—California and Texas, April 2009. MMWR Morb Mortal Wkly Rep 2009 ; 58 : 435-7.
3) Kamigaki T, Oshitani H. Epidemiological characteristics and low case fatality rate of pandemic (H1N1) 2009 in Japan. PLoS Curr Influenza 2009 : RRN1139.
4) Uyeki TM, et al. Low sensitivity of rapid diagnostic test for influenza. Clin Infect Dis 2009 ; 48 : e89-92.
5) Up to date on oseltamivir-resistant pandemic A (H1N1) 2009 influenza virus : January 2010. Wkly Epidemiol Rec 2009 ; 85 : 37-40.
6) WHO Weekly Surveillance. November, 2009.
7) Piedra PA, et al. Effects of oseltamivir on influenza-related complications in children with chronic medical conditions. Pediatrics 2009 ; 124 : 170-8.
8) Use of influenza A (H1N1) 2009 monovalent vaccine : Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. MMWR Recomm Rep 2009 ; 58 (RR-10) : 1-8.

Q98 水痘について，教えてください

A98

水痘は，水痘・帯状疱疹ウイルス（Varicella Zoster Virus）によってひき起こされる感染症で，健常な小児が感染すると発熱，発疹などの症状をきたしますが，免疫不全患者に感染すると重症化し，命を落とす患者さんもいます．ワクチンによる予防が最も重要です．

エビデンスレベルⅠ

回答者
齋藤昭彦

1. 疫学について教えてください

●水痘・帯状疱疹ウイルスはヘルペスウイルス族に属し，潜伏期間は多くの場合14～16日ですが，接触後10～21日の間で発症することもあるので，その期間は観察が必要です．

2. 臨床症状について教えてください

●水痘・帯状疱疹ウイルスによる感染症は，大きく2つの病態があります．1つは水痘（図1）で，もう1つは帯状疱疹（図2）です．

●水痘は，水痘・帯状疱疹ウイルスの初期感染で起こり，全身の痒みを伴う水疱様発疹が特徴的で，発疹は，水疱からかさぶたを伴うものまで，様々な病期のものが全身に出現することを特徴とします．また，頭皮に発疹が出現するのも特徴的です．

●合併症として，皮疹部位からの細菌感染，肺炎，急性小脳失調，脳炎，血小板低下などが挙げられます．児に比べ，成人での感染症はより重症化し，合併症の頻度が高いことが知られています．また，水痘罹患時にアスピリンを服用した患児にライ症候群の頻度が高いことが知られており，アスピリンの使用は控えます．免疫不全，特に化学療法中の患者が水痘に罹患すると重症化します．進行性の重症水痘は，皮疹の発現が止まらず，高熱，肝炎，脳炎，肺炎などを合併します．また，出血性水痘も起こります．

●帯状疱疹は，一度水痘に罹患した児の脊髄神経根に潜伏する水痘・帯状疱疹ウイルスが，再活性化して起こります．通常，1～3の神経根の分布に一致した神経部位に痛みを伴う水疱の集族をみます．免疫不全患者においては，播種性に広がり，重症化することもあります．

3. 伝播について教えてください

●水痘・帯状疱疹ウイルスは，空気感染で伝播する，伝染性の高い疾患です．すなわち，同じ空間を共有していると感染する可能性があります．帯状疱疹は，その局所が衣服などで覆われていれば，原則人に感染することはありません．しかしながら，免疫不全者の帯状疱疹は，播種する場合，空気感染する可能性があります．

4. 診断について教えてください

●診断は，臨床診断によることが多いですが，発疹の内容液などを用いて，PCR，蛍光抗体法などでの診断が最も感度の高い検査です．また，血清学的に水痘のIgMの上昇をみれば，急性感染症の診断がなされます．IgGは，過去の感染でも上昇しますが，2週間後に再検し，IgGの4倍以上の上昇をみれば，急性感染症の証明になります．

5. 治療について教えてください

●抗ウイルス薬の中でも，アシクロビルが水痘に効果があることが知られています．また，経口摂取が可能な患者では，より消化管からの吸収に優れたバラシクロビルも使用されます．その適応は，12歳より年長の児，慢性の皮膚，肺疾患をもつ児，長期のアスピリン療法を受けている児，ステロイド治療を受けている児などが挙げられます．水痘高力化免疫グロブリンは国内にありませんが，罹患歴のない免疫不全患者，妊婦，母親が分娩の5日前あるいは分娩後48時間までに水痘を発症した児などに対しては，使用することがあります．

6. ワクチンについて教えてください

●水痘の予防はワクチンによって可能です．水痘ワクチンは生ワクチンで，日本で作られたワクチンです．海外では広く使われており，疾患の制圧に成功していますが，残念ながら日本での接種率は30～40%と高くありません．ワクチンを接種できない免疫の弱い子どもたちを守るためには，その周りに暮らす家族と社会全体がワクチンを接種する必要があります．

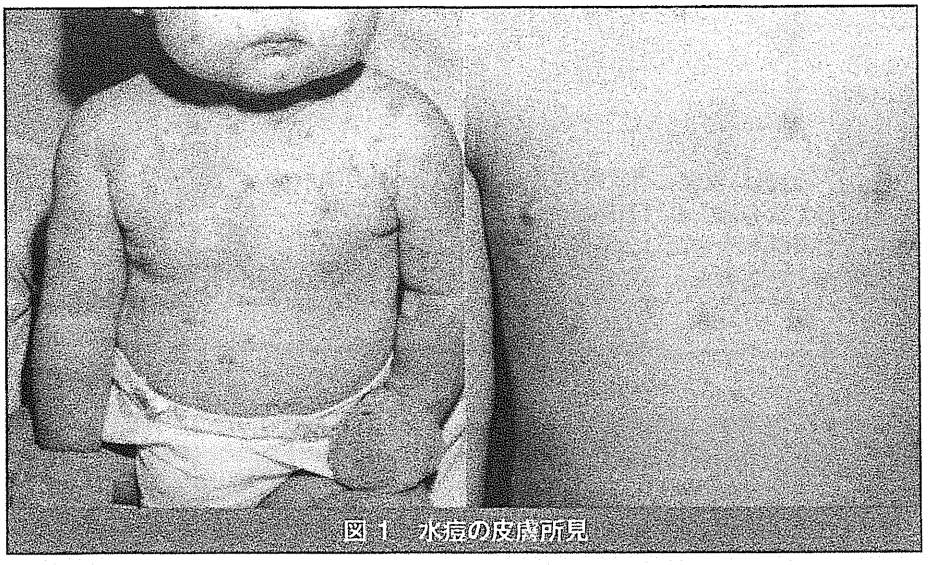


図1　水痘の皮膚所見

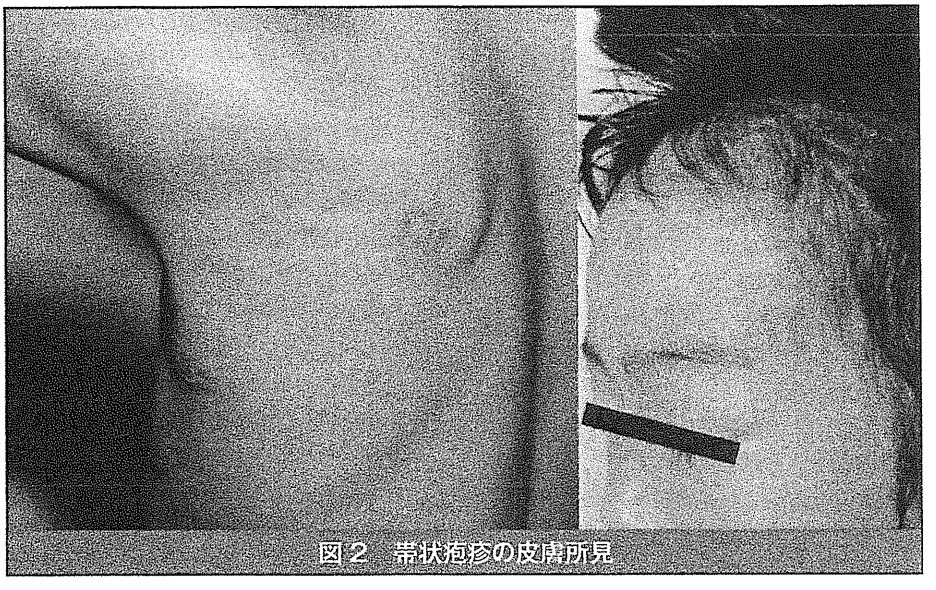
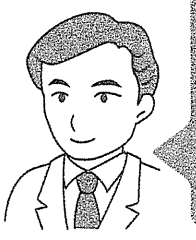


図2　帯状疱疹の皮膚所見



ワンポイントアドバイス

水痘は，伝染性の極めて高い疾患です．健康なお子さんが感染すると稀に合併症が起こることもあります．また，免疫の弱い患者さんが罹患すると重症化します．その予防には，ワクチンを接種することが重要です．

参考文献

1）American Academy of Pediatrics. Committee on Infectious Diseases. Report of the Committee on Infectious Diseases. In. Evanston, Ill.: American Academy of Pediatrics; 2009
2）Long SS, Pickering LK, Prober CG：Principles and practice of pediatric infectious diseases. 3rd ed. Philadelphia, PA: Churchill Livingstone/Elsevier; 2008
3）浅野喜造：水痘ワクチン．ウイルス 59:249-256, 2009

クロストリジウム・ディフィシル感染症

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- **病原体の特徴**: クロストリジウム・ディフィシル(*Clostridium difficile*)は、偏性嫌気性グラム陽性桿菌で、芽胞を形成する。産生する毒素には、toxin A および toxin B、さらに、binary toxin (actin-specific ADP-ribosyltransferase)が知られている。クロストリジウム・ディフィシル臨床菌株には、toxin A 陽性 toxin B 陽性株、toxin A 陰性 toxin B 陽性株、toxin A 陰性 toxin B 陰性株があり、toxin A 陽性 toxin B 陽性株および toxin A 陰性 toxin B 陽性株が腸炎・下痢症の原因となるとされる。Binary toxin は、毒素としての臨床的意義は明らかではないが、2000 年以降に欧米で流行株として知られる BI/NAP1/027 株が binary toxin を産生することから、注目されている。

- **感染症の特徴**:クロストリジウム・ディフィシルは、血液や創部から分離されることもあるが稀であり、ほとんどが消化管感染症である。抗菌薬や抗腫瘍薬の使用、加齢等により消化管細菌叢が撹乱された際に、クロストリジウム・ディフィシルを獲得し、さらに、免疫状態や基礎疾患の重症度等の因子が重なって、発症するとされている。クロストリジウム・ディフィシルは、健常人では消化管常在菌とは言い難いが、入院症例では無症候でクロストリジウム・ディフィシルを消化管保有する場合は少なくない。クロストリジウム・ディフィシル感染症(*Clostridium difficile* infection, CDI)は、医療関連感染としても重要で、国内外で院内アウトブレイク事例の報告がなされている。一方、最近では、市中感染例も少なくないことが報告されているため、他の原因が否定される場合は、外来下痢症例においても疑う必要がある。

- **感染経路**: 感染経路は接触感染である。患者のケアの際にプラスチック手袋の着脱を徹底することにより CDI 症例数が減少した事例、あるいは、環境の清掃に芽胞に有効な消毒薬を使用したことにより CDI 症例数が減少した病棟があった事例、さらには、直腸体温計を耳式体温計にしたところ CDI 症例数が減少した事例等から、医療スタッフの手指や医療機器を含めた医療環境が感染経路になっていると考えられる。

- **症状**: 軽度の下痢から、イレウスや消化管穿孔を伴う重症例、死亡例まで、症状に幅があることが特徴のひとつである。下痢、腹痛、血便、粘液便等の消化管症状のほか、白血球増多、発熱等の症状を伴うことがある。また、CDI は再発する症例が多い。再発には、治療中は芽胞の状態であったクロストリジウム・ディフィシルが治療終了後に

発芽・増殖する再燃、新しい菌株を獲得し発症する再感染があるが、どちらにしても、CDI 回復後に消化管細菌叢が回復しない状況で発症する。偽膜性大腸炎は、内視鏡、外科手術、あるいは剖検で消化管に偽膜形成が認められた場合に診断がつくもので、偽膜形成が認められない CDI 症例も多い。偽膜形成が認められた場合は、細菌学的検査を行わなくても CDI と診断できる。

- **細菌学的検査**:内視鏡等で偽膜形成が認められた場合を除き、CDI の診断には細菌学的検査が必要である。

  - 臨床的に CDI を疑う症例の糞便検体を検査することが原則で、消化管症状のない無症候キャリアや、治療経過のチェック目的で検査をすることは意義がない。分離培養目的であっても嫌気培養用の輸送容器を使用する必要はないが、信頼できる検査結果が得るには、十分量(5mL 程度)の検体量を採取することが重要である。
  - 現在、日本の臨床検査室で可能な検査は、糞便検体における毒素検出、クロストリジウム・ディフィシル分離培養、グルタメートデヒドロゲナーゼ(GDH)検出がある。毒素検出は、toxin A および toxin B を同時に検出する酵素抗体法による迅速検査キットが利用できるが、感度が高くないので、結果が陰性であっても CDI を否定できない場合が少なくない。クロストリジウム・ディフィシル分離培養は、嫌気培養さえ行うことができれば、手技的に難しくなく、感度が良好な検査法であるが、toxin A 陰性 toxin B 陰性菌株も、toxin B 陽性株と同様に分離されることに留意する必要がある。必要であれば、分離菌株において前述の酵素抗体法によるキットを用いて toxin A および／あるいは toxin B 産生性を調べることができる。ラテックス凝集反応による GDH 検出(C.D.チェック D-1)は、感度も特異度も高くないので、診断検査として単独での使用は勧められない。
  - 現在、利用できる検査法では、酵素抗体法による毒素検出検査(迅速キット)と、分離培養を組み合わせて施行し、検査結果および臨床症状を総合して診断する。毒素検出結果が陰性で臨床的に CDI が疑われる症例や、アウトブレイク発生が疑われる場合は、特に分離培養検査を行うことが勧められる。
  - 酵素抗体法による GDH 検出を、毒素検出の低い感度を補う目的で毒素検出キットに組み込んだものも開発されている。すなわち、GDH 陽性で、毒素(toxin A および toxin B)陰性の検体の場合は、次のステップとして同検体で他の検査を行う必要があるが、GDH 陰性、毒素陰性の検体は CDI を否定できるという利点があげられる。また、real-time PCR や loop-mediated isothermal amplification (LAMP)等による遺伝子検出検査も、今後さらに評価され利用されていくものと考えられる。

- **治療法**: CDI の誘因と考えられる抗菌薬を中止し、経過をみる(図 治療フローチャート)。下痢により脱水や電解質異常が認められる場合は、輸液等の治療が必要である。

中毒性巨大結腸症(toxic megacolon)を誘因するので、ロペラミド等の消化管蠕動を抑制する薬剤使用は避けるべきである。また、無症候キャリアは治療を行わないことが原則である。

- 抗菌薬中止により症状において回復傾向が認められない場合や、増悪する場合は、メトロニダゾールあるいはバンコマイシンを経口で使用する。メトロニダゾールは CDI を含む偏性嫌気性菌感染症の治療には適応認可されていないが、安価であり、軽度ー中等度の下痢症・腸炎症例ではバンコマイシンと同様の治療効果があると報告されているために、小児や妊婦以外では、日本でも使用している医療機関は多い。米国では1日 500mg を 10-14 日間処方される[1]。メトロニダゾールは、下痢便から固形便になると消化管で吸収されるために、消化管内での薬剤濃度は低くなる。バンコマイシンは、重症例 (125 mg/日を 10-14 日間)、重篤な合併症を伴う症例(~500 mg/日)の治療に使用される。バンコマイシンは静注しても消化管内で充分な薬剤濃度が得られないため、経口摂取、胃管経由あるいは、注腸によって使用する。メトロニダゾールにしてもバンコマイシンにしても消化管細菌叢の撹乱をきたすため、長期間処方しないことが原則である。
- メトロニダゾールやバンコマイシン以外では、抗菌スペクトラムが狭く消化管細菌叢を撹乱しにくい薬剤、クロストリジウム・ディフィシル毒素に吸着する薬剤、再発予防のためのモノクロナル抗体等が開発され海外で臨床試験が行われている。また、繰り返す再発例には、健康人ドナーからの糞便を「移植」する試みも行われている[2]。プロバイオティクスは消化管細菌叢のリカバーを期待して使用されるが、現在のところ、CDI の治療や再発予防にルーチンに使用する意義を裏付けるエビデンスは不十分とされる[3]。
- 近年、緊急外科手術が必要となる劇症腸炎症例の報告が増加している。外科手術施行の迅速な判断が、死亡率に大きく関与すると報告されている[4]。

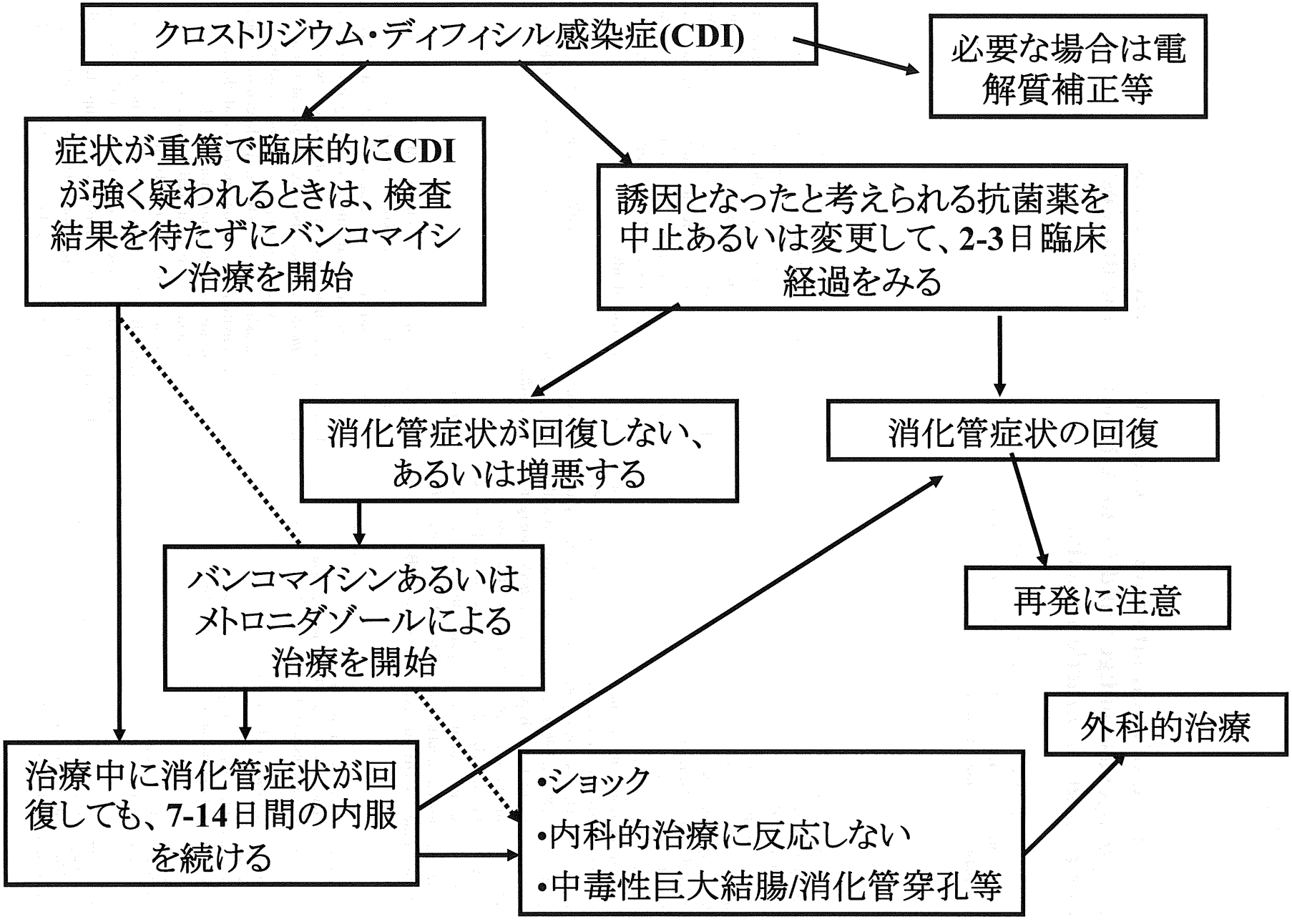
- **予防法**: 感染予防として、宿主側のリスクの軽減と、感染経路の遮断に分けられる。

  - 宿主側のリスクの軽減:CDI は抗菌薬関連下痢症として発症することが多く、加えて医療関連感染症でもあるため、医療機関全体で抗菌薬適正使用を行うことが重要である。理論的には、消化管細菌叢を撹乱しやすい抗嫌気性菌活性のある薬剤や広域スペクトラムの薬剤、胆汁排泄型の薬剤が問題となるが、すべての抗菌薬が CDI の誘因となりうる。消化管細菌叢は一度撹乱されると完全に回復するのに 2-3 ヶ月かかる場合が知られ、その間は再発しやすい時期であるため、抗菌薬の使用が必要な場合は薬剤の選択や下痢発症に留意する必要がある。
  - 感染経路の遮断:クロストリジウム・ディフィシルは芽胞の状態ではアルコール等の消毒薬が無効であるため、手指衛生は流水と石けんによる手洗いが基本であ

る[5]。速乾性擦り込み式アルコール製剤は手洗いの後に使用する。可能であれば、CDI 症例は個室隔離あるいはコーホーティングを行う。隔離解除は基本的には下痢症状がなくなり、発症前の排便状態に回復した時点とされる。メトロニダゾールやバンコマイシンが処方された場合は、内服終了時あるいは終了数日後まで経過をみることもある。接触予防策では、使い捨て手袋と使い捨てガウンの着脱を処置ごとに行うことを徹底し、特に排泄物の処理手順に注意する。アウトブレイク時では、CDI 症例の病室では、医療スタッフ、患者や家族が頻繁に接触する場所を定期的に消毒薬で清拭する。芽胞に有効で、安価で使いやすい消毒薬として次亜塩素酸ナトリウムが使用されることが多いが、塩素濃度として 1,000 ppm（6%市販製剤では 60 倍希釈）では、30 分間浸漬しないと芽胞に効果がないことに留意する。

文献:

1. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J and Wilcox MH. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol 31:431-455, 2010
2. van Nood E, Speelman P, Kuijper EJ and Keller JJ. Struggling with recurrent *Clostridium difficile* infections: is donor faeces the solution? Euro Surveill 142009
3. Dendukuri N, Costa V, McGregor M and Brophy JM. Probiotic therapy for the prevention and treatment of *Clostridium difficile*-associated diarrhea: a systematic review. Cmaj 173:167-170, 2005
4. Butala P, Divino CM. Surgical aspects of fulminant *Clostridium difficile* colitis. Am J Surg 200:131-135, 2010
5. Oughton MT, Loo VG, Dendukuri N, Fenn S and Libman MD. Hand hygiene with soap and water is superior to alcohol rub and antiseptic wipes for removal of *Clostridium difficile*. Infect Control Hosp Epidemiol 30:939-944, 2009


クロストリジウム・ディフィシル感染症(CDI)
必要な場合は電解質補正等
症状が重篤で臨床的にCDIが強く疑われるときは、検査結果を待たずにバンコマイシン治療を開始
誘因となったと考えられる抗菌薬を中止あるいは変更して、2-3日臨床経過をみる
消化管症状が回復しない、あるいは増悪する
消化管症状の回復
バンコマイシンあるいはメトロニダゾールによる治療を開始
再発に注意
外科的治療
治療中に消化管症状が回復しても、7-14日間の内服を続ける
•ショック
•内科的治療に反応しない
•中毒性巨大結腸/消化管穿孔等

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ORIGINAL ARTICLE

Evaluation of a simultaneous detection kit for the glutamate dehydrogenase antigen and toxin A/B in feces for diagnosis of *Clostridium difficile* infection

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Abstract Rapid detection kits for toxin A/B in feces are widely used as a diagnostic tool for *Clostridium difficile* infection (CDI). Their low sensitivity, however, has been considered a problem. In this study, we evaluated a new rapid diagnostic kit for simultaneous detection of the glutamate dehydrogenase (GDH) antigen and toxin A/B, *C. DIFF* QUIK CHEK COMPLETE. A total of 60 stool specimens from 60 patients with antibiotic-associated diarrhea were examined. Using *C. difficile* culture as the reference method, the GDH portion of this kit indicated a sensitivity, specificity, and negative predictive value of 100, 93.3, and 100%, respectively. The toxin A/B portion showed a sensitivity and specificity of 78.6 and 96.9%, respectively, compared to the culture results of toxin B-positive *C. difficile* (toxigenic culture). Of the 23 specimens that showed "dual positives" for GDH and toxin A/B, 22 were toxigenic culture positive, whereas *C. difficile* culture was negative in all the 28 specimens that showed "dual negatives" for GDH and toxin A/B. Of the nine "GDH-positive and toxin A/B-negative" specimens, six exhibited positive results by toxigenic culture. Results showing "dual positives" and "dual negatives" for GDH and toxin A/B can be reported as "true positive" and "true negative," respectively, whereas additional testing for confirmation, such as toxigenic culture, is required for specimens with discrepant results. Diagnostic algorithms, utilizing the simultaneous detection kit for GDH and toxin A/B as an initial screening test, may be useful for accurate and efficient diagnosis of CDI as well as the control of healthcare-associated infections.

Keywords *Clostridium difficile* infection · Glutamate dehydrogenase · Toxin A · Toxin B · Laboratory diagnosis · *Clostridium difficile* culture

Introduction

Clostridium difficile is a major causative agent of antibiotic-associated diarrhea and colitis. It can lead to a variety of clinical manifestations ranging from mild diarrhea to severe forms of intestinal illness including pseudomembranous colitis, ileus, toxic megacolon, and bowel perforation. *C. difficile* is a spore-bearing obligate anaerobe and is resistant to oxygen or antiseptics, depending on its spore status, thus making it possible for the pathogen to reside in the hospital environment for long periods and ultimately to cause healthcare-associated infections [1–4].

Toxins A and B have been well documented as major virulence factors of *C. difficile*. Toxin A-positive, toxin B-positive (A^+B^+) strains as well as toxin A-negative, toxin B-positive (A^-B^+) strains are known to cause diarrhea and colitis, and detection of toxin B in feces is important for the diagnosis of *C. difficile* infection (CDI). The traditional gold standard is a cytotoxin assay that detects the cytotoxicity of

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toxin B in fecal eluate by using cell culture, but this assay requires special equipment and techniques, making it unfeasible in many clinical laboratories [3, 4]. Rapid detection tests for toxin A/B by enzyme immunoassay (EIA) are widely used because of their ease of use and lower labor costs, but these tests appear to have a low sensitivity, which leads to concern about false-negative results [5–7]. Polymerase chain reaction (PCR) tests that detect the toxin B gene in stool samples may be rapid and more sensitive than EIA-based toxin detection, but it would be difficult for many ordinary clinical laboratories to employ these as routine diagnostic tests because acquisition of the instrument requires an initial large expenditure [7]. *C. difficile* culture followed by detection of a toxin-producing isolate (toxigenic culture) is considered the most sensitive diagnostic method. However, the turnaround time of at least 2–3 days until reporting of the final results is too long, and performing toxigenic culture on all the stool specimens submitted for *C. difficile* testing is labor intensive [8, 9].

Rapid detection tests for glutamate dehydrogenase (GDH), a cell-wall protein of *C. difficile*, have also been utilized as a diagnostic tool for CDI, although they provide no information about the toxigenicity. In contrast to the GDH detection tests by latex agglutination assay, those by EIA show a high sensitivity and negative predictive value, making them useful for rapid screening of *C. difficile* strains in feces. To make an accurate and efficient diagnosis of CDI, researchers have proposed testing algorithms, such as one that consists of an initial screening by EIA-based GDH detection, a rapid toxin detection test for GDH-positive specimens, and a final confirmatory test for GDH-positive/toxin-negative specimens by a cytotoxin assay, toxigenic culture, or PCR [3, 4, 8, 9].

Recently, an EIA-based rapid combination test kit for GDH and toxin A/B in feces, the *C. DIFF* QUIK CHEK COMPLETE (TECHLAB, Blacksburg, VA, USA) (*C. DIFF* COMPLETE), has become available in countries outside Japan and appears to be a promising aid in improving the accuracy and efficiency of CDI diagnosis [10–12]. In this study, we evaluated the *C. DIFF* COMPLETE in comparison with the other diagnostic methods including *C. difficile* toxigenic culture, a GDH detection test [ImmunoCard *C. difficile* (Meridian Bioscience, USA) (ImmunoCard)], and a toxin A/B detection test [TOX A/B QUIK CHEK (TECHLAB, USA) (TOX A/B)].

Materials and methods

Sample collection

A total of 60 fecal specimens were collected from 60 patients, who were hospitalized at Tokyo Metropolitan Geriatric Hospital (Tokyo, Japan) between October 2009 and January 2010 and diagnosed as having antibiotic-associated diarrhea (33 men and 27 women; age range, 54–100 years; mean, 82.9 years). All the patients consented to have their specimens used in this study. All samples examined were stored at 4°C for <72 h until *C. difficile* testing was performed.

Detection of the GDH and toxin A/B in fecal specimens

Simultaneous detection of the GDH and toxin A/B by *C. DIFF* COMPLETE, GDH detection by ImmunoCard, and toxin A/B detection by TOX A/B were performed in accordance with the manufacturers' instructions.

C. difficile culture

For *C. difficile* culture, fecal specimens were inoculated onto cycloserine–cefoxitin–mannitol agar (CCMA) (Nissui Pharmaceutical, Tokyo, Japan) and incubated for 48 h at 35°C under anaerobic conditions. The presence of the toxin A and toxin B genes of the recovered isolates was determined by PCR assay as previously described [13, 14]. For specimens that were "GDH-positive and toxin A/B-negative" by *C. DIFF* COMPLETE and "positive" by *C. difficile* culture, an additional *C. DIFF* COMPLETE test was performed on the isolates. Isolated colonies cultured on GAM agar (Nissui Pharmaceutical) for 48 h at 35°C under anaerobic conditions were suspended in the dilution buffer with turbidity equivalent to the McFarland standard no. 4 and tested by *C. DIFF* COMPLETE in accordance with the manufacture's protocol for fecal specimen testing.

Statistical analyses

As a significance test, the chi-square test with Yates' correction was performed. The significance level was set at $P < 0.05$.

Results

Clostridium difficile was isolated from 30 of the 60 fecal specimens. The GDH antigen portion of the *C. DIFF* COMPLETE test showed positive results in all 30 culture-positive specimens and negative results in 28 of the 30 culture-negative specimens, implying 100% sensitivity and 93.3% specificity. The negative predictive value was 100%. ImmunoCard exhibited positive results in 24 of the 30 culture-positive specimens and negative results in the remaining 36 specimens, indicating that its sensitivity and specificity were 80.0% (vs. *C. DIFF* COMPLETE, $P < 0.05$) and 100%, respectively (Table 1).

Table 1 Comparison of performance results for *C. DIFF* COMPLETE (GDH portion) and ImmunoCard compared to *Clostridium difficile culture*

		C. difficile culture		Sensitivity	Specificity	PPV	NPV	Correlation
		Positive	Negative	%	%	%	%	%
C. DIFF COMPLETE (GDH portion)	Positive	30	2	100 (30/30)	93.3 (28/30) *	93.8 (30/32) **	100 (28/28) **	96.7 (58/60) **
	Negative	0	28					
ImmunoCard	Positive	24	0	80.0 (24/30)	100 (30/30)	100 (24/24)	83.3 (30/36)	90.0 (54/60)
	Negative	6	30					

PPV, positive predictive value; NPV, negative predictive value

* $P < 0.05$, chi-square test with Yates' correction

** Not significant, chi-square test with Yates' correction

The results of toxin A/B detection by *C. DIFF* COMPLETE showed a correlation of 91.7% (55/60) with those by TOX A/B (data not shown). Compared with the culture results of toxin B-positive (i.e., A^+B^+ or A^-B^+) *C. difficile* (toxigenic culture), the sensitivity and specificity of the toxin A/B portion of *C. DIFF* COMPLETE were 78.6 and 96.9%, respectively, whereas those of TOX A/B were 71.4 and 93.8%, respectively (Table 2).

Of the 30 *C. difficile* isolates recovered from stool specimens, 19, 9, and 2 isolates were A^+B^+, A^-B^+, and toxin A-negative, toxin B-negative (A^-B^-) strains, respectively. Of the 19 fecal specimens from which A^+B^+ strains were isolated, 16 and 14 specimens exhibited positive results in the toxin A/B portion of *C. DIFF* COMPLETE and TOX A/B, respectively. Similarly, of the nine fecal specimens from which A^-B^+ strains were isolated, six specimens each exhibited positive results in respective assays, and of the two fecal specimens from which A^-B^- strains were isolated, one specimen gave a positive result in both tests (results of *C. DIFF* COMPLETE are shown in Table 3).

C. DIFF COMPLETE showed "dual positives" for GDH and toxin A/B in 23 fecal specimens. Toxin B-positive *C. difficile* strains were isolated from 22 of the 23 specimens, indicating a positive predictive value of 95.7% (an A^-B^- strain was isolated from the remaining 1 specimen). *C. DIFF* COMPLETE showed "dual negatives" for GDH and toxin A/B in 28 fecal specimens. All were negative for *C. difficile* culture, implying a negative predictive value of 100%. Of the nine specimens that showed "GDH-positive and toxin A/B-negative" results by *C. DIFF* COMPLETE, seven were positive for *C. difficile* culture (Table 3). By an additional *C. DIFF* COMPLETE test on the isolates, all the six toxin B-positive isolates (three A^+B^+ and three A^-B^+ isolates) showed "GDH-positive and toxin A/B-positive" results, whereas the one A^-B^- isolate indicated a "GDH-positive and toxin A/B-negative" result (data not shown).

Discussion

In this study, we evaluated *C. DIFF* COMPLETE, a new EIA-based rapid diagnostic tool for simultaneous detection of GDH and toxin A/B in a single device.

Using *C. difficile* culture as a reference, the GDH portion of the *C. DIFF* COMPLETE had a sensitivity of 100% (30/30), which was significantly higher than the sensitivity of 80% (24/30) observed in the ImmunoCard test. GDH detection in the *C. DIFF* COMPLETE test is mediated by anti-GDH polyclonal antibodies immobilized on the membrane (capture antibodies) and enzyme-labeled anti-GDH monoclonal antibodies (detector antibodies), whereas the ImmunoCard test utilizes anti-GDH polyclonal antibodies as both capture and detector antibodies. Such differences might contribute to the high sensitivity and specificity of GDH detection in *C. DIFF* COMPLETE.

For two specimens that were GDH positive by *C. DIFF* COMPLETE but culture negative, we cannot exclude the possibility of false-negative results of *C. difficile* culture. However, it was more strongly suspected that the GDH detection gave false-positive results because they exhibited negative results in all the other tests performed (i.e., GDH detection by ImmunoCard, toxin A/B detection by *C. DIFF* COMPLETE and TOX A/B), and neither of the two

Table 2 Comparison of performance results for *C. DIFF* COMPLETE (toxin portion) and TOX A/B compared to toxigenic culture

		A^+B^+/A^-B^+[a] *C. difficile* culture		Sensitivity %	Specificity %	PPV %	NPV %	Correlation %
		Positive	Negative					
C. DIFF COMPLETE (toxin portion)	Positive	22	1	78.6 (22/28)	96.9 (31/32)	95.7 (22/23)	83.8 (31/37)	88.3 (53/60)
	Negative	6	31		**	**	**	**
TOX A/B	Positive	20	2	71.4 (20/28)	93.8 (30/32)	90.9 (20/22)	78.9 (30/38)	83.3 (50/60)
	Negative	8	30					

PPV, positive predictive value; NPV, negative predictive value

** Not significant, chi-square test with Yates' correction

[a] A^+B^+, toxin A-positive, toxin B-positive; A^-B^+, toxin A-negative, toxin B-positive

Table 3 Results of the *C. DIFF* COMPLETE test and *C. difficile culture*

C. DIFF COMPLETE detecting		*C. difficile* culture			
		Positive			Negative
GDH	Toxin A/B	A^+B^+[a]	A^-B^+	A^-B^-	
Positive	Positive[b]	16[c]	6	1	0
Positive	Negative	3	3	1	2
Negative	Positive	0	0	0	0
Negative	Negative[b]	0	0	0	28
Total		19	9	2	30

[a] A^+B^+, toxin A-positive, toxin B-positive; A^-B^+, toxin A-negative, toxin-B-positive; A^-B^-, toxin A-negative, toxin B-negative

[b] A positive predictive value of "GDH-positive and toxin A/B-positive" results and a negative predictive value of "GDH-negative and toxin A/B-negative" results were 95.7% (22/23) and 100% (28/28), respectively

[c] Number of stool specimens

patients had received vancomycin or metronidazole for CDI before sample collection.

The toxin A/B portion of *C. DIFF* COMPLETE was able to detect both A^+B^+ and A^-B^+ strains, and had a detection rate of 78.6% (22/28) compared with the results of toxigenic culture, which was slightly higher than the rate of 71.4% (20/28) observed in the TOX A/B test (the difference was not statistically significant). Two A^+B^+ strains showed positive results in *C. DIFF* COMPLETE but negative results in TOX A/B, whereas the detection rate for A^-B^+ strains was the same for both tests (six of the nine specimens indicated positive results in each test).

Rapid detection EIA tests for toxin A/B have been reported to lack sufficient sensitivity [5–7]. In the present study, the toxin A/B portion of *C. DIFF* COMPLETE showed a relatively low sensitivity (78.6%), whereas the GDH portion indicated a sensitivity of 100%. Recent studies on *C. DIFF* COMPLETE have also described a low sensitivity of the toxin A/B portion (61.1–78.3%), in contrast to the high sensitivity of the GDH portion (97.6–100%) [10–12]. The specificity of toxin A/B detection by *C. DIFF* COMPLETE was high (96.9%), as reported in the articles described above (99.2–100%). Moreover, our study revealed a negative predictive value of 100% in GDH detection. Given such excellent sensitivity and negative predictive value of the GDH portion and the high specificity of the toxin A/B portion, using *C. DIFF* COMPLETE as the first-line screening test for CDI is considered reasonable.

Toxin B-positive *C. difficile* strains were isolated from 22 of the 23 fecal specimens that showed "dual positives" for GDH and toxin A/B by the *C. DIFF* COMPLETE test, implying a positive predictive value of 95.7%. The remaining 1 specimen from which an A^-B^- strain was isolated might have contained both toxin B-positive and A^-B^- strains, because TOX A/B as well as *C. DIFF* COMPLETE exhibited positive results for toxin A/B in this specimen. *C. difficile* culture was negative in all the 28 specimens that showed "dual negatives" for GDH and toxin A/B by the *C. DIFF* COMPLETE test, indicating a negative predictive value of 100%. Thus, results showing "dual positives" for GDH and toxin A/B strongly suggest "true positive," i.e., the presence of toxin B-positive

C. difficile strains in feces, whereas results showing "dual negatives" imply "true negative."

When the *C. DIFF* COMPLETE test shows "GDH-positive and toxin A/B-negative" results, it cannot be concluded that the nontoxic (A^-B^-) strain of *C. difficile* exists in feces. However, the GDH-positive result may alert clinicians to the possibility of the presence of toxigenic organisms as a result of the false-negative result for toxin A/B. Performing confirmatory tests only for the specimens with discrepant results (not for the dual-positive and dual-negative specimens) would alleviate the burden imposed on clinical microbiology laboratories and help improve the efficiency of accurate CDI diagnosis. Confirmatory testing by PCR on stool samples allows a rapid, simple, and accurate diagnosis [10–12], but the initial expenditure for purchase of the instrument is considerable. A cytotoxin assay may be also impracticable for many clinical laboratories because of the need for special equipment and techniques. Some researchers evaluated a combination of a toxin detection kit and *C. difficile* culture, in which the toxigenicity of a culture isolate was retested with the toxin detection kit [15, 16]. Such a diagnostic strategy may be feasible in many clinical institutions, although several days are required before results are reported.

C. difficile is important as a pathogen that frequently causes healthcare-associated infections. In the present study, 28 inpatients were diagnosed as having CDI during the study period of approximately 3.5 months. It is strongly suspected that some strains spread among these patients, although typing analysis on recovered isolates was not performed in this study. Diagnostic algorithms, utilizing the *C. DIFF* COMPLETE as an initial screening test, may contribute to the infection control of CDI by allowing us to make more accurate and rapid diagnosis of the infection.

References

1. Kelly CP, Pothoulakis C, LaMont JT. *Clostridium difficile* colitis. N Engl J Med. 1994;330:257–62.
2. Sato H, Kato H, Koiwai K, Sakai C. A nosocomial outbreak of diarrhea caused by toxin A-negative, toxin B-positive *Clostridium difficile* in a cancer center hospital. Kansenshogaku Zasshi. 2004;78:312–9.
3. Rupnik M, Wilcox MH, Gerding DN. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol. 2009;7:526–36.
4. Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). Infect Control Hosp Epidemiol. 2010;31:431–55.
5. Toyokawa M, Ueda A, Nishi I, Asari S, Adachi K, Annaka M, et al. Usefulness of immunological detection of both toxin A and Toxin B in stool samples for rapid diagnosis of *Clostridium difficile*-associated diarrhea. Kansenshogaku Zasshi. 2007;81:33–8.
6. Planche T, Aghaizu A, Holliman R, Riley P, Poloniecki J, Breathnach A, et al. Diagnosis of *Clostridium difficile* infection by toxin detection kits: a systematic review. Lancet Infect Dis. 2008;8:777–84.
7. Eastwood K, Else P, Charlett A, Wilcox M. Comparison of nine commercially available *Clostridium difficile* toxin detection assays, a real-time PCR assay for *C difficile* tcdB, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. J Clin Microbiol. 2009;47:3211–7.
8. Schmidt ML, Gilligan PH. *Clostridium difficile* testing algorithms: what is practical and feasible? Anaerobe. 2009;15:270–3.
9. Reller ME, Lema CA, Perl TM, Cai M, Ross TL, Speck KA, et al. Yield of stool culture with isolate toxin testing versus a two-step algorithm including stool toxin testing for detection of toxigenic *Clostridium difficile*. J Clin Microbiol. 2007;45:3601–5.
10. Sharp SE, Ruden LO, Pohl JC, Hatcher PA, Jayne LM, Ivie WM. Evaluation of the C.Diff Quik Chek Complete assay, a new glutamate dehydrogenase and A/B toxin combination lateral flow assay for use in rapid, simple diagnosis of *Clostridium difficile* disease. J Clin Microbiol. 2010;48:2082–6.
11. Quinn CD, Sefers SE, Babiker W, He Y, Alcabasa R, Stratton CW, et al. C. Diff Quik Chek Complete enzyme immunoassay provides a reliable first-line method for detection of *Clostridium difficile* in stool specimens. J Clin Microbiol. 2010;48:603–5.
12. Swindells J, Brenwald N, Reading N, Oppenheim B. Evaluation of diagnostic tests for *Clostridium difficile* infection. J Clin Microbiol. 2010;48:606–8.
13. Kato H, Kato N, Katow S, Meagawa T, Nakamura S, Lyerly DM. Deletions in the repeating sequence of the toxin A gene of toxin A-negative, toxin-B positive *Clostridium difficile* strains. FEMS Microbiol Lett. 1990;175:197–203.
14. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J Clin Microbiol. 1998;36:2178–82.
15. She RC, Durrant RJ, Petti CA. Evaluation of enzyme immunoassays to detect *Clostridium difficile* toxin from anaerobic stool culture. Am J Clin Pathol. 2009;131:81–4.
16. Wren M. *Clostridium difficile* isolation and culture techniques. Methods Mol Biol. 2010;646:39–52.

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Use of the loop-mediated isothermal amplification method for identification of PCR ribotype 027 *Clostridium difficile*

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The loop-mediated isothermal amplification (LAMP) assay detecting the *slpA* gene of *slpA* sequence type gc8 (*slpA-gc8*) was established for the identification of a hypervirulent *Clostridium difficile* strain, PCR ribotype 027. Of 107 isolates examined, 27 belonging to PCR ribotype 027 were all positive for the LAMP assay. The remaining 80 isolates were typed into 47 different PCR ribotypes other than type 027, and were negative for the LAMP assay with the exception of two isolates. The sensitivity and specificity of the LAMP method for identification of PCR ribotype 027 were 100 % and 98 %, respectively. The LAMP assay detecting *slpA-gc8* is a reliable tool for the identification of PCR ribotype 027 *C. difficile*. This simple and rapid method will contribute to detection of the hypervirulent strain.

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INTRODUCTION

Clostridium difficile is well known as an important cause of health-care-associated infection. The emergence of a *C. difficile* strain characterized as restriction endonuclease analysis (REA) type BI, PFGE type NAP1 and PCR ribotype 027 has been reported to be responsible in part for the epidemic in North America and Europe in the past decade (Kuijper *et al.*, 2008; Loo *et al.*, 2005; MacCannell *et al.*, 2006; McDonald *et al.*, 2005; Warny *et al.*, 2005). In addition, it has been documented that patients infected with this strain are more likely to develop severe disease (Barbut *et al.*, 2007; Goorhuis *et al.*, 2007). Since the epidemic by PCR ribotype 027 threatens to extend to the rest of the world including Asia (Gerding, 2010), early recognition of the emergence of the strain is valuable (Goorhuis *et al.*, 2007), especially in the countries or areas where the strain is not currently epidemic.

In previous studies, a system for typing *C. difficile* by sequencing the gene encoding the surface-layer protein A (*slpA*) was established and evaluated (Kato *et al.*, 2010; Killgore *et al.*, 2008). These reports documented that the typing results exhibited a high concordance with those obtained by PCR ribotyping; *slpA* sequence type gc8 corresponded to PCR ribotype 027 (Kato *et al.*, 2010; Killgore *et al.*, 2008). In the present study, we established a simple method for identification of PCR ribotype 027 by loop-mediated isothermal amplification (LAMP) detecting the *slpA* gene of *slpA* sequence type gc8 strains (*slpA-gc8*).

Abbreviations: CDT, binary toxin; LAMP, loop-mediated isothermal amplification; REA, restriction endonuclease analysis.

The GenBank/EMBL/DDBJ accession numbers (*slpA* sequence type) for the *slpA* genes reported in this study are AB249986 (gc8-01), AB257285 (gc8-02), AB257286 (gc8-03), AB461839 (gc8-05) and AB461840 (gc8-06).

METHODS

Bacterial strain. A total of 107 *C. difficile* isolates were examined. The reference strains of serogroups A (ATCC 43594), C (ATCC 43596), F (ATCC 43598), G (ATCC 43599) and H (ATCC 43600) (Delmée *et al.*, 1986) were obtained from the American Type Culture Collection. The 59 clinical *C. difficile* isolates used in this study were isolated from various hospitals in Japan. A collection of 42 isolates used for a previous study including strain US42 (REA type BI/PFGE type NAP1/PCR ribotype 027; Killgore *et al.*, 2008) and strain UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008) were tested as well.

PCR detecting the toxin genes. The non-repeating and repeating sequences of the toxin A gene (*tcdA*) were amplified by PCR with primer sets NK3–NK2 (Kato *et al.*, 1991) and NK9–NK11–NKV011 (Kato *et al.*, 1998, 1999), respectively. The isolates were identified as toxin A-negative when PCR with the primer set NK3–NK2 was negative (Kato *et al.*, 1991). When PCR by the NK3–NK2 primer set was positive and PCR by the NK9–NK11–NKV011 primer set generated an amplification product of approximately 1200 bp in size, the isolates were identified as toxin A-positive. When PCR by NK3–NK2 was positive and the product by PCR with NK9–NK11–NKV011 was approximately 700 bp in size, the isolates were identified as toxin A-negative (Kato *et al.*, 1998, 1999). When the isolates were positive for PCR by NK3–NK2 but the PCR product generated by NK9–NK11–NKV011 was neither approximately 1200 bp nor 700 bp in size, toxin production by the isolates was determined. The presence of the non-repeating sequences of the toxin B gene (*tcdB*) was examined by PCR using primer set NK105–NK104 (Kato *et al.*, 1998). PCR detection of the gene encoding the binding component of binary toxin (CDT) was performed as previously described (Stubbs *et al.*, 2000).

Determination of toxin production by isolates. *C. difficile* was cultured anaerobically in brain–heart infusion broth (Becton

Dickinson) for 5 days. Toxin A was detected by an enzyme immunoassay kit, *C. difficile* toxin A test (Oxoid). Toxin B was detected by a culture assay using Vero cells and *C. difficile* goat anti-toxin B serum was used for the neutralization test (Kato *et al.*, 1998).

Typing of isolates. Typing of isolates by sequencing *slpA* was carried out as previously described (Kato *et al.*, 2010). Isolates were assigned to different major types when they had 20 or more amino acid differences, and to subtypes (01–06) when they had fewer than 20 such differences. PCR ribotyping was performed by the modified methods of Stubbs and others as previously described (Kato *et al.*, 2010; Stubbs *et al.*, 1999).

Detection of *slpA-gc8* by LAMP. For DNA extraction for LAMP, *C. difficile* was inoculated on Brucella agar supplemented with vitamin K_1 and haemin (Kyokuto Pharmaceutical) for 1–7 days anaerobically. A single colony was suspended in 50 µl TES (50 mM Tris hydrochloride, pH 8.0; 5 mM EDTA; and 50 mM NaCl). The suspension was heated at 95 °C for 15 min and then centrifuged at 15 000 *g* for 2 min, and the resultant supernatant was used as a template for the LAMP assay. The six primers used for the LAMP were derived from the *slpA* gene of strain US42, which was identified as REA type BI, PFGE type NAP1, PCR ribotype 027 and *slpA* sequence type gc8-01 (GenBank accession no. AB249986) (Kato *et al.*, 2010; Killgore *et al.*, 2008). The outer primers used were gc8002a-F3 (5′-GCTCTCCAGCAGAGGGAG-3′) and gc8002-B3 (5′-AGTTCCA-TCAACTAAACCAAAC-3′); the inner primers were gc8002b-FIP (5′-TTGGAGCTGTATTTTTTGCTCCCGCAATAAAAGTAGCTACAAG-TAG-3′) and gc8002-BIP (5′-GTCTATGTCAGATGTATTTGATAC-ATAAGTTTCACAGCAGTTTCAGT-3′); and the loop primers were gc8002-FL (5′-TACCAGCATTTTCGACTTCACC-3′) and gc8002-BL (5′-GCTTTTACAGATTCA-3′). The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical) according to the manufacturer's instructions. Two microlitres of DNA template was added to a total volume of 25 µl buffer consisting of 5 pM of each of the outer primers, 40 pM of each of the inner primers and 20 pM of each of the loop primers. Amplification was performed at 62 °C for 60 min. The increased turbidity was monitored by a real-time turbidimeter, LA-320C (Eiken Chemical). The turbidity was calculated based on the ratio of light intensity (the intensity of light received by the photodiode/the emitted light intensity). The threshold of LAMP positive for the turbidimeter was defined as 0.1.

Determination of the sequences of *tcdC*. The sequences of *tcdC* were determined by sequencing the PCR product with primer set C1–C2 as described previously (Spigaglia & Mastrantonio, 2002). The primer set NK3–Lok1 (Braun *et al.*, 1996; Kato *et al.*, 1991) was used for sequencing *tcdC* of isolate DJNS 7-18. The sequences obtained were compared to those of strain VPI 10463 and strain US42 as well as to the sequences previously described (MacCannell *et al.*, 2006) and those in the GenBank database. The comparative analysis was performed by GENETYX-MAC version 12.2.3.

RESULTS

Typing results

Sequencing of the *slpA* gene resulted in 24 major types and 54 subtypes for the 107 isolates tested. Of the 107 isolates, 29 were typed as *slpA* sequence type gc8, and five different subtypes (*slpA* sequence types gc8-01, gc8-02, gc8-03, gc8-05 and gc8-06) were found among these 29 isolates (Table 1). Types gc8-01, gc8-02, gc8-03 and gc8-06 differed from each other by 1 nt, involving a single deduced amino acid. The *slpA* sequence of one isolate, DJNS 7-18, was found to differ from that of gc8-01 by eight deduced amino acids (*slpA* sequence type gc8-05). Among the 107 isolates, 48 different PCR ribotypes were identified. Of the 28 isolates belonging to gc8-01, gc8-02, gc8-03 or gc8-06, 27 were typed as PCR ribotype 027 (Fig. 1). One isolate of *slpA* sequence type gc8-01 (JND 10-61) was typed as a different PCR ribotype from PCR ribotype 027 (PCR ribotype 027r; Fig. 1), while its PCR banding pattern showed two band differences from that of 027. Isolate DJNS 7-18 and the remaining 78 isolates were typed into 46 different PCR ribotypes, banding patterns of which were distinct from that of PCR ribotype 027. The epidemiologically important PCR ribotypes 001, 002, 014, 017, 078, trf and smz (Barbut *et al.*, 2007; Goorhuis *et al.*, 2008; Kato *et al.*, 2010; Killgore *et al.*, 2008) were included in the 46 PCR ribotypes.

Detection of *slpA-gc8* by LAMP

slpA-gc8 was detected by LAMP in all 29 isolates of *slpA* sequence type gc8 including JND 10-61 and DJNS 7-18 (Fig. 2; Table 1). LAMP was performed on DNA extracted from strain US42, which was incubated for 1 or 7 days. The time for *C. difficile* culture to extract DNA did not affect the LAMP reaction. In a comparison of the LAMP results in 28 isolates identified as PCR ribotypes 027 and 027r (*slpA* sequence types gc8-01, gc8-02, gc8-03 and gc8-06), the amplification reaction in DJNS 7-18 (PCR ribotype tkm0718/*slpA* sequence type gc8-05) was slow (Fig. 2). LAMP was performed on 78 isolates of types other than *slpA* sequence type gc8 in a 90 min reaction, with negative results (Table 1). The sensitivity and specificity of the LAMP method for identification of PCR ribotype 027 were 100 % and 98 %, respectively.

slpA sequence type gc8 isolates recovered in Japan

Of seven *slpA* sequence type gc8 isolates recovered in Japan, six (subtypes gc8-01 and gc8-06) were toxin A-positive, toxin B-positive and CDT-positive. Sequencing analysis of the six isolates including JND 10-61 detected *tcdC* identical with that of strain US42 and published sequences (MacCannell *et al.*, 2006). Isolate DJNS 7-18 (subtype gc8-05) was toxin A-negative, toxin B-positive and CDT-positive. In the DJNS 7-18 isolate, PCR using the NK3–NK2 primer set was positive but no amplification was obtained by PCR using the NK9–NK11–NKV011 primer set. This isolate was tested by enzyme immunoassay for toxin A production, with a negative result, and toxin B was detected by a cell culture assay with a positive neutralization reaction. When the sequences of *tcdA* and *tcdC* were compared to those registered in the GenBank database, DJNS 7-18 had deletions in *tcdA* and *tcdC*; the deletions were the same in location and size as those of strain 8864 (GenBank accession no. AJ011301). Isolate DJNS 7-18 was

Table 1. Typing results and LAMP results for identification of PCR ribotype 027

slpA sequence major type	*slpA* sequence subtype	PCR ribotype	LAMP results	No. of isolates tested from:			Total no. of isolates studied
				Japan	North America and Europe*	ATCC	
gc8	gc8-01	027	+	4	16		20
	gc8-01	027r†	+	1			1
	gc8-02	027	+		2		2
	gc8-03	027	+		4		4
	gc8-05	tkm0718	+‡	1			1
	gc8-06	027	+	1			1
Other types§			−	52	21	5	78
Total				59	43	5	107

*A collection of 42 isolates used for a previous study (Killgore *et al.*, 2008) and strain UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008).
†The banding pattern of JND 10-61 (PCR ribotype 027r) differed from that of type 027 by two bands.
‡The LAMP reaction in DJNS 7-18 was slow.
§A total of 78 isolates were typed into 23 *slpA* sequence major types, 49 *slpA* sequence subtypes and 45 PCR ribotypes.

recovered from a patient who suffered from pseudomembranous colitis in 2008.

DISCUSSION

In the present study, all PCR ribotype 027 isolates tested were successfully identified by LAMP assay detecting the *slpA-gc8* gene. The isolates typed as 47 different PCR ribotypes other than type 027 were all negative for LAMP with the exception of two isolates, JND 10-61 and DJNS 7-18. In JND 10-61, which was typed as *slpA* sequence gc8-01, the PCR ribotype pattern differed from that of type 027 by two bands and its *tcdC* sequence was the same as that of strain US42. More studies are needed to identify this isolate as a strain related to the hypervirulent strain. DJNS 7-18 was a toxin A-negative, toxin B-positive, CDT-positive isolate with the same deletions in *tcdA* and *tcdC* as those of strain 8864 (Lyerly *et al.*, 1992; Rupnik *et al.*, 1998). Since strain 8864 was not available in the present study, PCR ribotyping and *slpA* sequence typing results for strain 8864 could not be compared to those for DJNS 7-18. DJNS 7-18 was unique among our collection of clinical isolates in Japan. Additional studies using more clinical isolates are warranted to determine the significance of isolates typed as *slpA* sequence type gc8 but not as PCR ribotype 027.

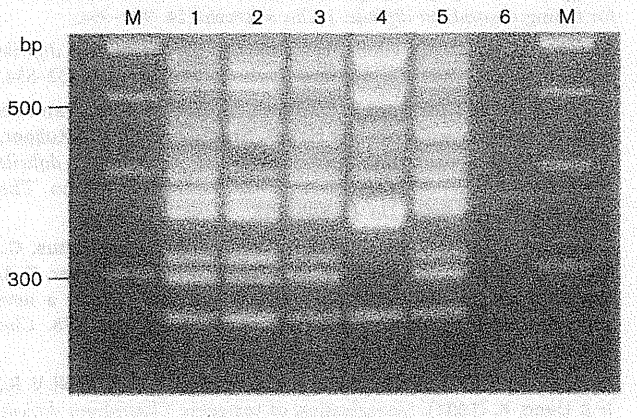

Fig. 1. PCR ribotype patterns of representative isolates typed as *slpA* sequence type gc8. Lanes: 1, DJNS 5-23 (*slpA* sequence type gc8-01); 2, JND 10-61 (gc8-01); 3, JND 8-64 (gc8-06); 4, DJNS 7-18 (gc8-05); 5, US42 (gc8-01); 6, negative control; M, 100 bp ladder as a molecular size marker. Strain US42 was used as the reference strain of PCR ribotype 027.

PCR ribotype 027 has been reported to be responsible for multiple outbreaks and a major portion of the increase in *C. difficile* infection rates in North America and Europe (Kuijper *et al.*, 2008; Pépin *et al.*, 2004; Loo *et al.*, 2005; McDonald *et al.*, 2005). It has been documented that patients infected with the PCR ribotype 027 strain are more likely to have severe disease and to have been specifically treated with metronidazole or vancomycin (Barbut *et al.*, 2007). Goorhuis *et al.* (2007) demonstrated that clear trends were observed for more severe diarrhoea, higher attributable mortality and more recurrences in patients infected with PCR ribotype 027 than in those infected with other types (Goorhuis *et al.*, 2007). The significant pathogenicity of this strain, such as robust toxin production or significantly more spore formation (Merrigan *et al.*, 2010; Warny *et al.*, 2005), may contribute to the severity and wide spread of *C. difficile* infection caused by the strain. These reports indicate that PCR ribotype 027 is more likely to cause outbreaks with high morbidity and mortality. Particularly in the countries or areas where the strain is not currently predominant, such as in Japan (Kato *et al.*, 2007; Sawabe *et al.*, 2007), earlier recognition of outbreaks caused by the strain will provide beneficial information for public health centres, leading to alerts of

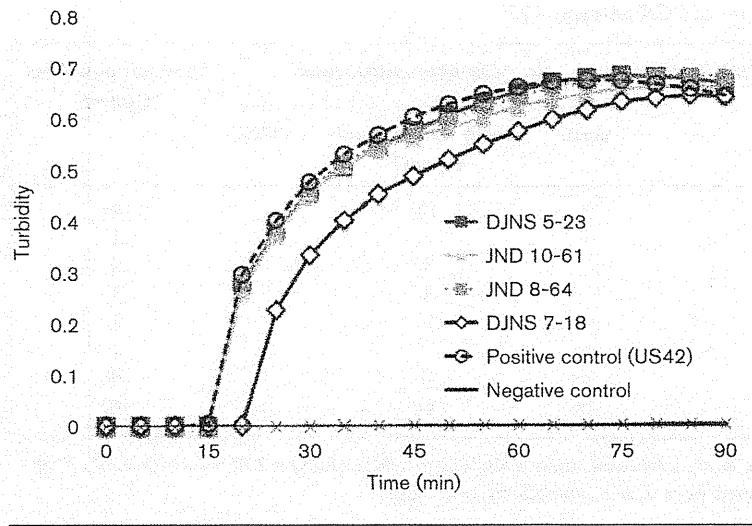

Fig. 2. Real-time turbidity of LAMP amplification products for representative isolates typed as *slpA* sequence type gc8. Strain US42 was used as a positive control.

the emergence of the strain to health-care facilities covered by the public centres. The LAMP assay detecting *slpA-gc8* may be valuable for monitoring trends in the prevalence of *C. difficile* infection caused by PCR ribotype 027. Since the LAMP method is simple to perform and does not require any special equipment, such as a thermal cycler or electrophoresis system, it is accessible to even small laboratories of local public health centres as well as of health-care facilities.

The LAMP assay detecting *slpA-gc8* proved to be a reliable tool for the identification of PCR ribotype 027 in the present study. The method may be applied to the direct detection of *slpA-gc8* from stool specimens without a culture step. In the present study, one stool specimen from a patient who suffered from pseudomembranous colitis caused by PCR ribotype 027 was available (Kato *et al.*, 2007); detection of *slpA-gc8* was performed on DNA extracted directly from the stool specimen as previously described (Kato *et al.*, 2005), with a positive result (data not shown). Further analysis using a large number of stool specimens is required to evaluate the method for the direct detection of PCR ribotype 027 strains from stool specimens.

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REFERENCES

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E., Poxton, I. on behalf of the European Study Group on Clostridium difficile (ESGCD) (2007). Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin Microbiol Infect* **13**, 1048–1057.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M. & von Eichel-Streiber, C. (1996). Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* **181**, 29–38.

Delmée, M., Laroche, Y., Avesani, V. & Cornelis, G. (1986). Comparison of serogrouping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* **24**, 991–994.

Gerding, D. N. (2010). Global epidemiology of *Clostridium difficile* infection in 2010. *Infect Control Hosp Epidemiol* **31** (Suppl. 1), S32–S34.

Goorhuis, A., Van der Kooi, T., Vaessen, N., Dekker, F. W., Van den Berg, R., Harmanus, C., van den Hof, S., Notermans, D. W. & Kuijper, E. J. (2007). Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. *Clin Infect Dis* **45**, 695–703.

Goorhuis, A., Bakker, D., Corver, J., Debast, S. B., Harmanus, C., Notermans, D. W., Bergwerff, A. A., Dekker, F. W. & Kuijper, E. J. (2008). Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis* **47**, 1162–1170.

Kato, N., Ou, C. Y., Kato, H., Bartley, S. L., Brown, V. K., Dowell, V. R., Jr & Ueno, K. (1991). Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. *J Clin Microbiol* **29**, 33–37.

Kato, H., Kato, N., Watanabe, K., Iwai, N., Nakamura, H., Yamamoto, T., Suzuki, K., Kim, S. M., Chong, Y. & Wasito, E. B. (1998). Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* **36**, 2178–2182.

Kato, H., Kato, N., Katow, S., Maegawa, T., Nakamura, S. & Lyerly, D. M. (1999). Deletions in the repeating sequences of the toxin A gene of

toxin A-negative, toxin B-positive *Clostridium difficile* strains. *FEMS Microbiol Lett* **175**, 197–203.

Kato, H., Yokoyama, T., Kato, H. & Arakawa, Y. (2005). Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. *J Clin Microbiol* **43**, 6108–6112.

Kato, H., Ito, Y., van den Berg, R. J., Kuijper, E. J. & Arakawa, Y. (2007). First isolation of *Clostridium difficile* 027 in Japan. *Euro Surveill* **12**, E070111–E070113.

Kato, H., Kato, H., Ito, Y., Akahane, T., Izumida, S., Yokoyama, T., Kaji, C. & Arakawa, Y. (2010). Typing of *Clostridium difficile* isolates endemic in Japan by sequencing of *slpA* and its application to direct typing. *J Med Microbiol* **59**, 556–562.

Killgore, G., Thompson, A., Johnson, S., Brazier, J., Kuijper, E., Pépin, J., Frost, E. H., Savelkoul, P., Nicholson, B. & other authors (2008). Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. *J Clin Microbiol* **46**, 431–437.

Kuijper, E. J., Barbut, F., Brazier, J. S., Kleinkauf, N., Eckmanns, T., Lambert, M. L., Drudy, D., Fitzpatrick, F., Wiuff, C. & other authors (2008). Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveill* **13**, 433–439.

Loo, V. G., Poirier, L., Miller, M. A., Oughton, M., Libman, M. D., Michaud, S., Bourgault, A. M., Nguyen, T., Frenette, C. & other authors (2005). A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med* **353**, 2442–2449.

Lyerly, D. M., Barroso, L. A., Wilkins, T. D., Depitre, C. & Corthier, G. (1992). Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. *Infect Immun* **60**, 4633–4639.

MacCannell, D. R., Louie, T. J., Gregson, D. B., Laverdiere, M., Labbe, A. C., Laing, F. & Henwick, S. (2006). Molecular analysis of *Clostridium difficile* PCR ribotype 027 isolates from Eastern and Western Canada. *J Clin Microbiol* **44**, 2147–2152.

McDonald, L. C., Killgore, G. E., Thompson, A., Owens, R. C., Jr, Kazakova, S. V., Sambol, S. P., Johnson, S. & Gerding, D. N. (2005). An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl J Med* **353**, 2433–2441.

Merrigan, M., Venugopal, A., Mallozzi, M., Roxas, B., Viswanathan, V. K., Johnson, S., Gerding, D. N. & Vedantam, G. (2010). Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. *J Bacteriol* **192**, 4904–4911.

Pépin, J., Valiquette, L., Alary, M. E., Villemure, P., Pelletier, A., Forget, K., Pépin, K. & Chouinard, D. (2004). *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* **171**, 466–472.

Rupnik, M., Avesani, V., Janc, M., von Eichel-Streiber, C. & Delmée, M. (1998). A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* **36**, 2240–2247.

Sawabe, E., Kato, H., Osawa, K., Chida, T., Tojo, N., Arakawa, Y. & Okamura, N. (2007). Molecular analysis of *Clostridium difficile* at a university teaching hospital in Japan: a shift in the predominant type over a five-year period. *Eur J Clin Microbiol Infect Dis* **26**, 695–703.

Spigaglia, P. & Mastrantonio, P. (2002). Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J Clin Microbiol* **40**, 3470–3475.

Stubbs, S. L., Brazier, J. S., O'Neill, G. L. & Duerden, B. I. (1999). PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* **37**, 461–463.

Stubbs, S., Rupnik, M., Gibert, M., Brazier, J., Duerden, B. & Popoff, M. (2000). Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* **186**, 307–312.

Warny, M., Pépin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., Frost, E. & McDonald, L. C. (2005). Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**, 1079–1084.

【総説】

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