る．その際，多くの候補の中からより有望なバイオ医薬品候補をより迅速・的確にピックアップし，効率的な医薬品開発の推進を図るためには，基礎研究から臨床への橋渡し，特に探索的臨床研究（トランスレーショナルリサーチ：TR）を適正に実施することが，きわめて重要な要素になる．ちなみに，現在までに，スムーズな開発に成功したバイオ医薬品は，生命科学分野での学問的解明や技術開発の進歩の成果を合理的にかつ効率よく医療への応用に結びつけたものである[30]．TRを適正に実施するためには，科学的要素の効率的連携や集約による科学的妥当性の検証，社会的理解や認知を得るための倫理的妥当性の提示，経済的妥当性の検討などを，効率よく，かつ合理的に行う必要がある．また，一般的な方向づけや留意事項等を示したガイドラインなど規制環境の整備その他サポート体制の整備もきわめて重要な基盤的要素になる．

医薬品開発におけるTR移行への科学的妥当性とは「それまでの基礎研究，応用研究，開発研究などで得られた知見で当該医薬品の品質，安定性，安全性及び予想される有効性や性能の面からTRを行うことの妥当性を明らかにする」ことであると考えられる．もちろん，これは，医薬品承認申請時におけるようなフルセットのデータを必ずしも求めているわけではない．TR開始に際して考慮すべき科学的要素の具体的事項例としては，①医薬品の種類，②起原または発見の経緯及び類似の製品の使用状況，③製造方法の妥当性，④分子特性/細胞特性解析，⑤品質評価・管理，⑥安定性評価，⑦感染性物質に対する安全性確保を含む安全性，⑧薬効の裏付け，⑨体内動態等，⑩臨床計画（臨床目的・適用法等），⑪学術的知見・経験の多寡，⑫臨床応用後の情報収集体制，等が挙げられる．製造方法から体内動態等までについて最小限必要と思われるデータを適切に集め，結果を総括してTRに入ることの妥当性を示すことが求められる．

重要なことは，TR開始に必要なデータの種類やその程度は個別製品ごとに異なること，個々の医薬品についての試験の実施や評価に際しては，品質・安全性や予想される有効性の評価と臨床的有用性に関する探索研究という目的をふまえ，その時点の学問の進歩を反映した合理的根拠に基づき，ケース・バイ・ケースの原則で柔軟に対応することである．したがってポイントは，個別製品ごとに，品質・安全性等に関する必要なデータをいかに合理的に集め，いかに適正なタイミングでTRの開始にもっていくかということにある．これにはさまざまな立場の専門家や機関の共同作業，連携がきわめて重要であると思われる．TRの例ということではないが，これまでに承認されたタンパク質性バイオ医薬品の開発事例を見ると，基礎研究の段階でその特性や作用機構が有効性・安全性と関連して解明されている程度や各関係者間の連携の緊密度に応じて，短期間で臨床応用に進んでいる[30]．

バイオ医薬品の開発を一層適正に効率よく推進させるためには，適切なタイミングで基礎・応用・開発研究からTRへと移行することが重要であるが，その際，TRやさらに1歩進んだ臨床試験へ移行することの客観的妥当性を確保するため，規制環境の整備も重要である．バイオ医薬品のTRや臨床試験への移行や実施法に特化した公的なガイドラインは，

わが国には存在しない．しかし先に述べたように遺伝子治療や細胞治療に関しては，特に先端的な医療であることから，臨床研究や治験を行うにあたって科学的妥当性や倫理的妥当性を示すうえでの基本的考え方，必要なデータや留意事項などの詳細が厚生労働省より通知されているので，これらがTRや臨床試験への移行や実施のための指針として利用できると思われる．

細胞基材由来タンパク質性のバイオ医薬品に関しては，新規製品開発に際して必要な技術的要件に関する国際調和（ICH（International Conférence on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use）：日米EU医薬品規制調和国際会議）ガイドラインがある．TRのためのものではないが，基本的な考え方やTRに合理的に利用できる事項についてはこれらを参考にすると良いと思われる[7,8]．具体的には，「細胞基材」，「遺伝子安定性」，「ウイルス安全性」，「製品の安定性」，「特性解析・品質規格」，「非臨床安全性」についての指針が示されている[31-36]．これらのガイドラインは，細胞基材由来タンパク質性医薬品，すなわち，組換え医薬品や細胞培養医薬品の品質・安全性とその恒常性の確保を図るために必要な要素のうち，①目的物質の製造工程の明確化とその妥当性の評価・検証，特に，遺伝子発現構成体の構築や解析及び細胞培養中の安定性，細胞基材の由来，調製及びバンク化とその特性解析及び品質評価，細胞基材の安定性，②製品の十分な特性解析や品質評価，製品の規格及び試験方法や原材料の試験及び工程内管理試験の設定と実施，ロット間の品質恒常性の立証，③安定性試験と評価，④ウイルス安全性評価，⑤非臨床における安全性評価，等の要素について作成されたものである．また，各種バイオ医薬品の中では，タンパク質性の医薬品は実用化が最も進み，特性解析，品質，安全性試験や評価のあり方についても多くの知見や経験が集積されている．今後のバイオ医薬品の開発においては，これらICHガイドラインやこれまでに得られた多くの知見や経験の集積をいかに有効に合理的に活用するかが非常に重要なポイントになると思われる．

この他，より一般的には「医薬品の臨床試験のための非臨床安全性試験の実施時期についてのガイドライン」（平成10年11月13日医薬審第1019号）[37]，さらに臨床試験の実施要領に関しては「医薬品の臨床試験の実施の基準（GCP：Good Clinical Practice）」（平成9年3月27日厚生省令第28号）[38] を参考にすべきである．これらのガイドラインを製品の種類や特徴，その他の要素を勘案しながら，合理的に活用すると良いと思われる．

バイオ医薬品に関連する研究成果を医療に応用する場合，一般に，薬剤を業として，すなわち企業活動として開発するのか，医療行為の一環として研究用薬剤を調達し，医療実践をするのかにより規制環境が異なる．上述のガイドラインは業として開発する場合を念頭においたものである．一方，薬剤を自己調達して医療行為として実施する場合には薬事法的規制はない．医療従事者が科学的妥当性の観点と倫理的妥当性の観点で判断し，患者に対するインフォームド・コンセントを含めて，責任をもって医療行為の一環として行うことである．しかし，患者の立場や社会的観点から見た場合，適正な水準や条件を充たし

ている必要があることは言うまでもない.

より一層適正なTRの推進と新薬の早期創出に向けて，厚生労働省では薬事法改正の検討が進められ，平成14年7月には改正薬事法が成立した．従来は，医師が主体となって行う臨床研究のデータを承認申請に利用することはできず，また，医師の依頼があっても製薬企業が未承認の薬物を製造し提供することは認められていなかった．しかし，今回の改正により，製薬企業が医師に対して臨床研究用の薬物などを製造・提供することが可能になり，また，臨床研究の成果を一定条件のもとで製薬企業の申請データの一部に採用することも可能となった．この際，研究を担当する医師からの治験届の提出，GCPに基づいた研究の実施が条件として要求され，臨床研究に対して倫理性と科学性を持たせつつ，臨床研究の成果を新薬開発に迅速に反映させられる仕組みが構築されることとなった.

6 おわりに

バイオ医薬品の臨床試験にとって重要なことは，個々の医薬品の製造方法，有効成分の種類・特性，製品の品質，安定性，毒性，薬理，体内動態などを熟知したうえで，臨床試験の目的を明確に立て，試験実施の正当性と理由を説明し，被験者選択基準，被験者への説明と同意取得法，対象とする効能・効果と評価項目・判定基準，症例数，臨床用量，投与経路，投与頻度，投与期間等を設定することである．こうした個別製品の特徴，特殊性に応じた適切な臨床試験計画のもと，試験を実施し，的確な臨床観察，試験の進行状況のモニタリング，監査，記録の作成，結果の適正な解析を行い，報告する必要がある．遺伝子治療や細胞治療等の先端的医療技術の適用にあたっては，予測できない事態の発生とその対応に備えて，感染症発生の有無等を長期追跡調査すること，臨床記録・製品記録・製品及びドナーや被験者由来検査試料をしかるべき期間保存する措置，その他関連情報の積極的収集なども必要である.

今後，画期的なバイオ医薬品を効率よく創製していくためには，基礎研究の成果をもとに，基盤となる最新の科学技術をいかに適切に用いてデータを集積し，得られた知見や関連情報をいかに効率よく連携させ，合理的に集約していくかが課題である．こうしたアプローチにより，新規遺伝子発現産物の生命現象での役割や生物学的作用に関する理解の深まりと連動して，有用物質の臨床応用への道がよりスムーズに開けるものと期待される．これからはさらに医療技術的にも新しく，また，経済的妥当性，社会的理解・認知，倫理的妥当性の面でもあらかじめ用意された答はない，いわば新たな挑戦となるものが次々と出てくることが予測される．これらについては，関係者がより優良な医薬品や適正な医療技術を患者に1日でも早く提供するという観点に立ち，英知を結集して，医療の進歩と課題の克服にあたることが重要であると思われる.

参考文献

1）厚生省薬務局審査課長，生物製剤課長通知，組換えＤＮＡ技術を応用して製造される医薬品の承認申請に必要な添付資料の作成について，薬審第243号，昭和59年.
2）厚生省薬務局審査第一課長，審査第二課長，生物製剤課長通知，細胞培養技術を応用して製造される医薬品の承認申請に必要な添付資料の作成について，薬審１第10号，昭和63年.
3）文部科学省，厚生労働省，遺伝子治療臨床研究に関する指針，告示第１号，平成14年.
4）厚生労働省医薬局長通知，遺伝子治療用医薬品の品質及び安全性の確保に関する指針の改正について，医薬発第0329004号，平成14年.
5）厚生労働省医薬局長通知，薬事法施行規則の一部を改正する省令等の施行について（細胞組織医薬品及び細胞組織医療用具に関する取扱いについて），（（別添）細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方），医薬発第266号，平成13年.
6）厚生省医薬安全局長通知，ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保について，（（別添２）ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針），医薬発第1314号，平成12年.
7）早川堯夫，石井（渡部）明子，医薬品研究，33(11)，693-728（2002）.
8）早川堯夫，衛研報告，117，1-38（1999）.
9）早川堯夫，"バイオ医薬品の品質・安全性評価"（早川堯夫，山崎修道，延原正弘編），エル・アイ・シー，東京，2001，p.101-122.
10）早川堯夫，山口照英，新見伸吾，内田恵理子，押沢正，医薬品研究，20，735-759（1989）.
11）T. Hayakawa, ed.by Y.-Y. Chiu and J.L. Gueriguian, "Scientific and Regulators Aspects of Drug Biotechnology", Marcel Dekker Inc., New York, 1990, p.468-498及び引用文献.
12）森本和滋，内田恵理子，川崎ナナ，アハメド・アブド・サイード，徳永裕司，春日井勲，早川堯夫，医薬品研究，25，405-425（1994）.
13）森本和滋，内田恵理子，川崎ナナ，アハメド・アブド・サイード，徳永裕司，春日井勲，早川堯夫，医薬品研究，25，501-523（1994）.
14）川崎ナナ，早川堯夫，"バイオ医薬品の品質・安全性評価"（早川堯夫，山崎修道，延原正弘編），エル・アイ・シー，東京，2001，p.255-284.
15）M. Ohta, N. Kawasaki, S. Itoh, S. Hyuga, M. Hyuga and T. Hayakawa, *Biologicals*, 30, 235-244（2002）.
16）T. Hayakawa, M. Ohta and N. Kawasaki, *Pharmaeuropa*, Special Issue（Biologicals beyond 2000, Challenge for Quality Standard in an Evolving Field）, 87-102（2000）及び引用文献.
17）H. Schellekens, *Nature Reviews, Drug Discovery*, 1, 457-462（2002）及び引用文献.
18）NIH Report, *Human Gene Therapy*, 13, 3-14（2002）.
19）早川堯夫，水口裕之，医薬ジャーナル，37，83-88（2001）.
20）M. Cavazzana-Calvo, S. Hacein-Bey, G. de Saint Basile, F. Gross, E. Yvon, P. Nusbaum, F. Selz, C. Hue, S. Certain, J.L. Casanova, P. Bousso, F.L. Deist, A. Fischer, *Science*, 288, 669-672（2000）.
21）M.A. Kay, C.S. Manno, M.V. Ragni, P.J.Larson, L.B.Couto, A.McClelland, B. Glader, A.J. Chew, S.J.Tai, R.W. Herzog, V. Arruda, F. Johnson, C. Scallan, E. Skarsgard, A.W. Flake, K.A. High, *Nat. Genet.*, 24, 257-261（2000）.

22) I. Baumgartner, A. Pieczek, O. Manor, R. Blair, M. Kearney, K. Walsh, J.M.Isner, *Circulation*, 97, 1114-1123 (1998).
23) 早川堯夫, “バイオ医薬品の品質・安全性評価”（早川堯夫, 山崎修道, 延原正弘編), エル・アイ・シー, 東京, 2001, p.341-350.
24) 早川堯夫, “バイオ医薬品の品質・安全性評価”（早川堯夫, 山崎修道, 延原正弘編), エル・アイ・シー, 東京, 2001, p.397-419.
25) 早川堯夫, 内田恵理子, 黒澤努, 白倉良太, 医薬品研究, 31, 791-817 (2000).
26) 上田正次, “バイオ医薬品の品質・安全性評価”（早川堯夫, 山崎修道, 延原正弘編), エル・アイ・シー, 東京, 2001, p.443-452.
27) 早川堯夫, 豊島聡, 山口照英, 川西徹, 衛研報告, 119, 1-26 (2001).
28) 國田智, 鍵山直子, “バイオ医薬品の品質・安全性評価”（早川堯夫, 山崎修道, 延原正弘編), エル・アイ・シー, 東京, 2001, p.469-486.
29) 山内一也, “バイオ医薬品の品質・安全性評価”（早川堯夫, 山崎修道, 延原正弘編), エル・アイ・シー, 東京, 2001, p.453-465.
30) 早川堯夫, 石井（渡部）明子, 医学のあゆみ, 200, 539-543 (2002).
31) 厚生省医薬安全局審査管理課長通知, 生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）製造用細胞基材の由来, 調製及び特性解析について, 医薬審第873号, 平成12年.
32) 厚生省医薬安全局審査管理課長通知, 組換えDNA技術を応用したタンパク質生産に用いる細胞中の遺伝子発現構成体の分析について, 医薬審第3号, 平成10年.
33) 厚生省医薬安全局審査管理課長通知,「ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス安全性評価」について, 医薬審第329号, 平成12年.
34) 厚生省医薬安全局審査管理課長通知, 生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）の安定性試験について, 医薬審第6号, 平成10年.
35) 厚生労働省医薬局審査管理課長通知, 生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）の規格及び試験方法の設定について, 医薬審第571号, 平成13年.
36) 厚生省医薬安全局審査管理課長通知,「バイオテクノロジー応用医薬品の非臨床における安全性評価」について, 医薬審第326号, 平成12年.
37) 厚生省医薬安全局審査管理課長通知, 医薬品の臨床試験のための非臨床安全性試験の実施時期についてのガイドラインについて, 医薬審第1019号, 平成10年（一部改正, 医薬審第1831号, 平成12年).
38) 厚生大臣, 医薬品の臨床試験の実施の基準に関する省令, 厚生省令第28号, 平成9年.

doi:10.1016/j.ymthe.2003.09.001

METHOD

Detection of Replication-Competent Adenoviruses Spiked into Recombinant Adenovirus Vector Products by Infectivity PCR

Akiko Ishii-Watabe,[1,†] Eriko Uchida,[2] Akiko Iwata,[2,3] Ryuji Nagata,[2] Kouei Satoh,[3] Kejun Fan,[4] Mitsuhiro Murata,[4] Hiroyuki Mizuguchi,[2] Nana Kawasaki,[1] Toru Kawanishi,[1] Teruhide Yamaguchi,[2] and Takao Hayakawa[5]

[1]*Division of Biological Chemistry and Biologicals and* [2]*Cellular and Gene Therapy Products, and* [5]*National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[3]*Institute of Saitama Red Cross, 1370-12 Takaogi, Hidaka, Saitama 350-1213, Japan*
[4]*JSR Corporation, 25 Miyukigaoka, Tsukuba 305-0841, Japan*

[†]*To whom correspondence and reprint requests should be addressed. Fax: +81-3-3700-9084. E-mail: watabe@nihs.go.jp.*

The presence of replication-competent adenovirus (RCA) in clinical lots of adenovirus vectors raises a variety of safety concerns. To detect RCA in adenovirus vector products, the cell culture/cytopathic effect (CPE) method has generally been preferred. However, it is difficult to evaluate the amount of RCA clearly and quantitatively by this method. In addition, the cell culture/CPE method requires large-scale cell culturing and a substantial amount of time. For the purpose of establishing a method to detect RCA more sensitively and rapidly, we developed the infectivity PCR, a hybrid method that combines the infectivity assay and quantitative PCR. This method allows RCA to be quantified by real-time quantitative PCR using primers and a probe designed for E1 DNA. By infectivity PCR, 1 pfu of RCA spiked into 10^9 particles of adenovirus vectors could be detected. In contrast, CPE was observed in the cells infected with 10^4 pfu of RCA spiked into 10^9 particles of adenovirus vectors. The glass-beads method was suitable for extracting DNA rapidly from the RCA-infected cells. These results showed that infectivity PCR combined with the glass-beads-based DNA extraction method was useful for the detection of RCA in adenovirus vector products.

Key Words: replication-competent adenovirus, adenovirus vector, infectivity PCR

INTRODUCTION

Recombinant adenovirus vector is one of the most promising vectors available for human gene therapy. In fact, adenovirus vector-based gene therapies now account for 26.9% of all clinical gene-therapy protocols [1]. Considerable efforts have been made to improve the potency of adenovirus vectors to make them more useful for gene therapy, e.g., regulation of target-cell specificity by modifying fiber protein [2–7], application of cassettes that enable the regulation of gene expression [8,9], and reduction of immunogenicity by deleting all viral genome sequences [10]. However, there have been few studies investigating the establishment of a system to ensure the safety of these vectors.

The vast majority of adenovirus vectors are constructed by inserting the therapeutic genes in place of the essential viral E1 sequence in the adenovirus. The generation of E1-deleted adenovirus vectors relies on the complementation functions present in HEK293 cells into whose genome E1 DNA has been inserted. However, HEK293 cells are prone to the generation of replication-competent adenovirus (RCA) as a result of recombination events between the vector DNA and the integrated adenovirus sequences present in the cells [11]. The presence of RCA in adenovirus vector products raises the possibilities of adenovirus infection, unintended vector replication due to the presence of wild-type helper function, and exacerbation of host inflammation response [12]. Because it is extremely difficult to avoid completely the emergence of RCA in adenovirus vector products by means of the current production technique, examining the level of RCA in each lot of adenovirus vector products is important [12]. In addition, examining for the presence of RCA in the patients who have been administrated adenovirus vectors

Copyright © The American Society of Gene Therapy
1525-0016/03 $30.00

doi:10.1016/j.ymthe.2003.09.001

is important to test for viral shedding during the clinical study.

As a method to detect RCA in adenovirus vector products, the cell culture/cytopathic effect (CPE) assay has generally been used [13,14]. In the cell culture/CPE assay, the vector products are infected into cells, the RCA are amplified, and the CPE induced by the RCA is observed. By this method, the presence of RCA is judged by microscopic observation, and thus the results may not always be accurate and quantitative. In addition, this method requires large-scale cell culturing and a substantial amount of time. As a sensitive method to detect viral DNA, PCR is thought to be useful [11,15]. However, because the infectivity of the viral DNA cannot be measured by PCR, the cell culture/CPE assay is still recommended by the FDA [12]. For all of the above reasons, there is need for a more sensitive, quantitative, and rapid method for the detection of RCA.

In the present study, we established an infectivity PCR method for detecting RCA. Infectivity PCR is a hybrid method that combines the best features of the infectivity assay and PCR. By this method, the virus is allowed to replicate in the cell culture, as in the traditional cell culture/CPE assay, and the amount of virus replicated in the cells is determined by quantitative PCR rather than by observing CPE. For efficient extraction of the DNA from RCA-infected cells, a novel glass-bead method was developed. Our results demonstrated that the infectivity PCR method combined with glass-beads-based DNA extraction was superior to the classical cell culture/CPE method for detecting RCA.

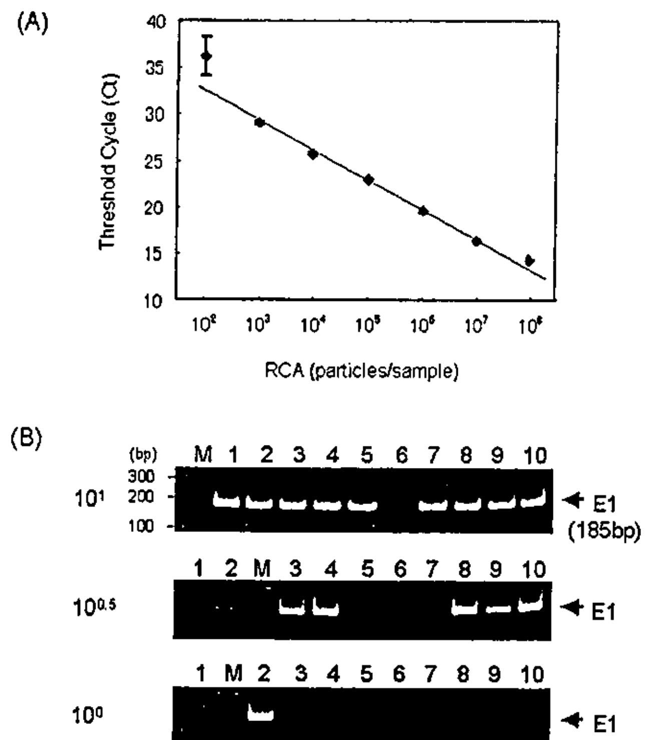


FIG. 1. Detection of RCA by PCR. Viral genome DNA was extracted from serial log dilutions of RCA and amplification of each sample was performed by (A) real-time quantitative PCR or (B) nested PCR. (A) Standard curve for the determination of RCA quantity generated from the amplification plot of real-time quantitative PCR. Data are the means ± SD of triplicate amplifications. (B) Detection of a low copy number of RCA by nested PCR (n = 10). The particle numbers in the samples were 10^1, $10^{0.5}$, and 10^0. M, molecular weight marker.

RESULTS

Quantification of RCA by Real-Time Quantitative PCR

As a first step in establishing an infectivity PCR method, we developed a real-time quantitative PCR that can quantify the copy number of the RCA genome. We designed four pairs of primers and probes for the detection of E1 DNA, which is included in the RCA genome but not in the adenovirus vectors. Among them, the Ad5dE1-1035F and Ad5dE1-1105R primers and the Ad5dE1-1058TM probe worked well for quantifying the RCA genome. Fig. 1A shows the standard curve with the starting quantity of RCA on the x axis and the threshold cycle (Ct denotes the PCR cycle at which the threshold line intercepts the amplification curve) on the y axis. The threshold cycle and the log-transformed concentration showed a high, inverse correlation in a linear fusion from 10^8 to 10^3 particles.

Detection of RCA by Nested PCR

Since the nested PCR method is known to be suitable for detecting low concentrations of DNA, we applied it here to detect the DNA extracted from 10^1, $10^{0.5}$, or 10^0 particle(s)/tube of RCA. When nested PCR was performed in 10 tubes, the amplification succeeded in the ratios of 9/10 for 10^1 particles, 5/10 for $10^{0.5}$ particles, and 1/10 for 10^0 particles (Fig. 1B). Although DNA concentration cannot be quantitatively determined by nested PCR, it can be estimated from the hit rate of the amplification. This estimation assumes that, when low concentrations of samples are transferred from a stock tube to PCR tubes, viral particles will not necessarily be present in all the reaction tubes. If the PCR is optimized, then as little as one copy of DNA can be amplified, and the relationship between the hit rate in the PCR and the average copy number in the PCR tubes can be calculated as follows: 1/10 for 0.105, 2/10 for 0.233, 3/10 for 0.357, 4/10 for 0.511, 5/10 for 0.693, 6/10 for 0.916, 7/10 for 1.20, 8/10 for 1.61, and 9/10 for 2.30 [16,17]. In our experiments, half of the extracted DNA was subjected to the nested PCR; therefore, the hit rate of the PCR and the RCA copy number were 1/10 for 0.5, 5/10 for 1.58, and 9/10 for 5. Although the hit rate in this experiment seemed to be slightly lower than the theoretical values, this does not

Copyright © The American Society of Gene Therapy

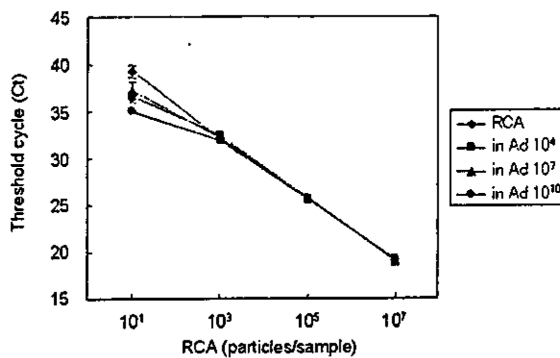


FIG. 2. Detection of RCA spiked into adenovirus vectors by real-time quantitative PCR. 10^1, 10^3, 10^5, or 10^7 particles of RCA were spiked into 0, 10^4, 10^7, or 10^{10} particles of adenovirus vectors (AdHM4LacZ). Viral DNA was extracted from each sample, and E1 DNA was detected by real-time quantitative PCR. Data are the means ± SD of triplicate amplifications. The background Ct (Ad without any RCA) was 39.1 for 10^4 particles of adenovirus vectors, 39.2 ± 1.6 for 10^7 particles, and 35.2 ± 0.04 for 10^{10} particles.

necessarily invalidate the results, since DNA may have been lost during the DNA extraction, and the detection limit may have been higher than one copy. These results demonstrated that the nested PCR could be used for the detection of lower concentrations of RCA and that the detection limit might be fewer than 10^1 particles.

Measurement of RCA in Adenovirus Vector Products

Because, in practice, it is often necessary to detect very slight amounts of RCA in high concentrations of adenovirus vectors, we next tried to quantitate the amount of RCA spiked into adenovirus vectors. We extracted viral genome DNA from 10^1, 10^3, 10^5, or 10^7 particles of RCA spiked into 10^4, 10^7, or 10^{10} particles of adenovirus vectors and then measured the amount of RCA by real-time quantitative PCR. As shown in Fig. 2, when the RCA concentration was higher than 10^3 particles/sample, the amount of RCA could be measured without major interference by coexisting adenovirus vectors. However, when the spiked RCA concentration was 10^1 particles, the Ct value seemed to be lowered by the presence of adenovirus vectors. From these results, although RCA was thought to be detectable even in the presence of adenovirus vectors, the adenovirus vectors used in this experiment might have contained a certain amount of E1 DNA. We therefore examined several lots of adenovirus vectors for the presence of E1 DNA by nested PCR. When 10^{10} particles of adenovirus vectors were subjected to the nested PCR, E1 DNA was detected in all seven lots tested. When 10^9 particles of adenovirus vectors were tested, E1 DNA was detected in four of the seven lots tested (data not shown).

Because we could not distinguish whether the E1 DNA detected in adenovirus vectors was derived from RCA or HEK293 cells, we tested for the presence of HEK293 cell-derived DNA in the vector products by PCR. For this purpose, we used primers designed for the sequence of the pregnancy-specific glycoprotein (PSG) gene, in which the adenovirus E1 gene is inserted into the HEK293 cell genome [18]. In all lots examined, PSG DNA was detected in 10^{10} particles of adenovirus vectors, meaning that the adenovirus vector products contained HEK293 cell-derived DNA as impurities (Fig. 3A). E1 DNA was barely detected in 10^{10} particles of adenovirus vectors (Fig. 3B). These results showed that adenovirus vectors can be subjected to the direct measurement of RCA by PCR, although the results are complicated by the presence of residual HEK293 cell DNA. To separate RCA from HEK293 cell-derived DNA, we attempted to establish an infectivity PCR method for the measurement of RCA in adenovirus vectors. In the infectivity PCR assay, RCAs are expected to be selectively amplified in the cells that support their growth.

Comparison of the Amount of RCA in Cells and Cultured Medium from RCA-Infected Cell Culture

To determine a suitable source for the PCR template in infectivity PCR, we compared the amounts of E1 DNA in the cells and cultured medium from the RCA-infected cell culture. We infected HeLa cells with serially diluted RCA and cultured them. We extracted DNA from the cells and cultured media and then subjected it to real-time quantitative PCR. In this experiment, we used one-third of the cells or one-hundredth of the supernatant from each dish for DNA extraction. A 100-fold higher amount of RCA was

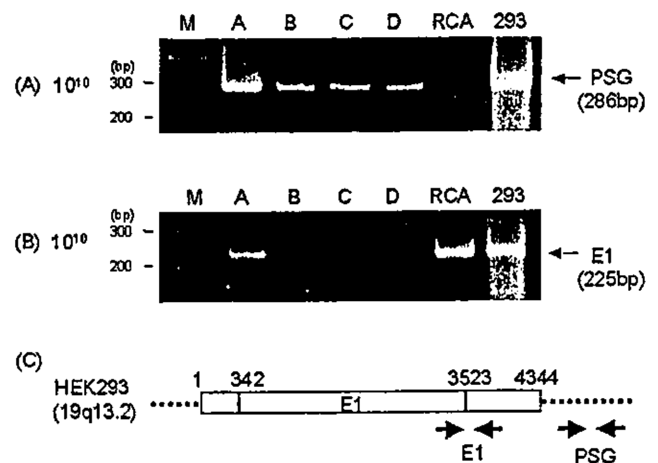
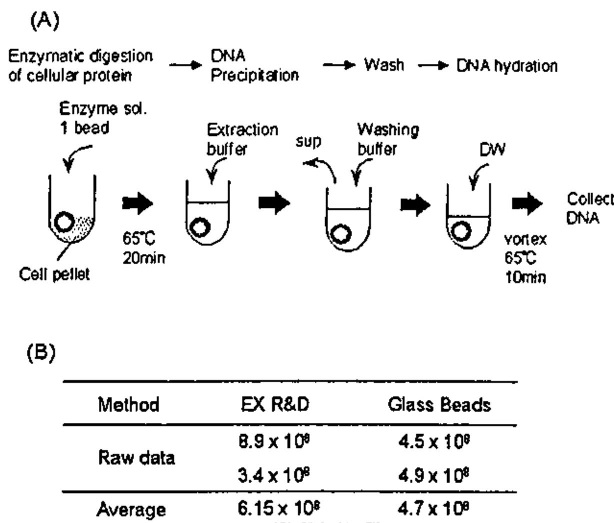


FIG. 3. Detection of pregnancy-specific glycoprotein (PSG) or E1 DNA in adenovirus vectors by PCR. Viral DNA was extracted from 10^{10} particles of adenovirus vectors and then (A) 293 cell-derived pregnancy-specific glycoprotein DNA or (B) E1 DNA was detected by PCR. DNA from HEK293 cells was used as a positive control. Lane A, AdHM4LacZ (Lot 0516); lane B, AdHM10LacZ-3 (Lot 0529); lane C, AdHM10LacZ-4 (Lot 0529); lane D, AdHM10LacZ-5 (Lot 0529), lane RCA, replication-competent adenoviruses; lane 293, genomic DNA extracted from HEK293 cells; lane M, molecular weight marker. (C) E1 DNA inserted into the chromosome of HEK293 cells and the positions of the PCR primers used are shown.

Copyright © The American Society of Gene Therapy

doi:10.1016/j.ymthe.2003.09.001



Method	EX R&D	Glass Beads
Raw data	8.9 x 10^8	4.5 x 10^8
	3.4 x 10^8	4.9 x 10^8
Average	6.15 x 10^8	4.7 x 10^8

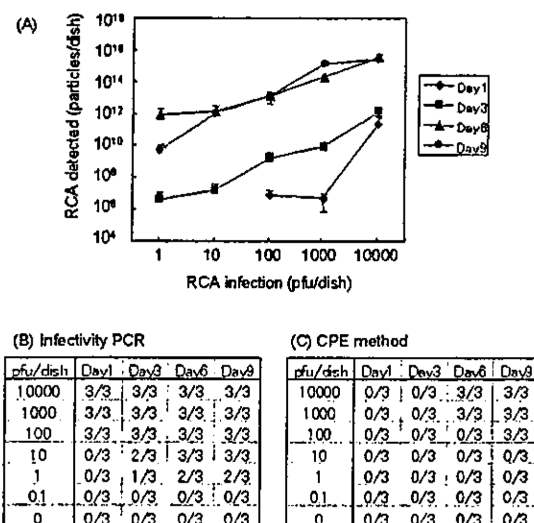
FIG. 4. DNA extraction using glass beads. (A) Procedure of DNA extraction from cell pellets using glass beads. (B) Comparison of the efficiency of DNA extraction. DNA was extracted from RCA-infected HeLa cell lysate by the EX-R&D reagent or glass-beads method. The copy number of E1 DNA in each sample was determined by real-time quantitative PCR.

detected in the DNA extracted from the cells compared with that from the cultured medium (data not shown). Therefore, we considered the cells to be a suitable source for further examination of the propagation of RCA.

In the preparation of DNA from RCA-infected cells, cellular genomic DNA caused high viscosity and disturbed the subsequent PCR. However, cellular genomic DNA could be digested by nuclease after freezing and thawing without damaging the viral DNA, because the viral DNA is protected by capsid proteins. By this procedure, we could extract viral DNA from more than 1×10^6 cells and use it as a template for the PCR, although this procedure was laborious. We considered that glass beads, which were initially developed for extracting cellular DNA, might be used to simplify this step, and therefore we examined a glass-beads-based DNA extraction method (Fig. 4A). The protocol for the glass-beads method is simple and less time-consuming than extracting DNA after freezing and thawing followed by nuclease treatment. For comparing the efficiency of DNA extraction using glass beads to that using the SMI TEST EX-R&D, we extracted DNA from cells that contain the same amount of RCA. We used the SMI TEST EX-R&D as an example of a method that can extract DNA with high efficiency [19]. As shown in Fig. 4B, we detected equal amounts of RCA in DNA extracted by these two methods, meaning that glass beads are useful for extracting DNA rapidly, with an efficiency similar to that of the previous method.

Infectivity PCR for the Detection of RCA

For examining the infectivity PCR, we used RCA in the absence of adenovirus vectors as a preliminary experiment. HeLa cells were infected with serial log-diluted RCA (0, 0.1, 1, 10, 100, 1000, 10,000 pfu) in triplicate and cultured. After culturing them for 1, 3, 6, or 9 days, we harvested the cells and extracted the DNA using glass beads. We then measured the amount of RCA in each sample by real-time quantitative PCR. As shown in Figs. 5A and 5B, we detected RCA in all of the triplicate samples that had been infected with more than 1 pfu of RCA. RCA was not detected in the samples that had been infected with 0.1 pfu of RCA. Therefore, we concluded that at least 1 pfu of RCA was detectable by this assay. In parallel with this infectivity PCR assay, we observed the cells by microscopy and tested for CPE (Fig. 5C). On day 6, we observed weak signs of CPE in the cells that had been infected with 1000 pfu of RCA, and we clearly observed CPE in the cells that had been infected with 10,000 pfu of RCA. On day 9, we observed slight CPE in the cells that had been infected with 100 pfu of RCA, and we clearly observed CPE in the cells that had been infected with 1000 or 10,000 pfu of RCA. These results showed that the sensitivity of infectivity PCR was 100 or 1000 times higher than that of the CPE assay. In addition, by the infectivity PCR method, RCA could be detected at an earlier time point than by the CPE assay. Since A549 cells are often used for CPE assay, we also examined infectivity PCR using A549 cells. The viability of the cells decreased later than day 6, and it was difficult to test the CPE. The amount of RCA amplified in A549 cells tended to be lower than that in HeLa cells(data not shown). Therefore, we used HeLa cells for further examination.



(B) Infectivity PCR

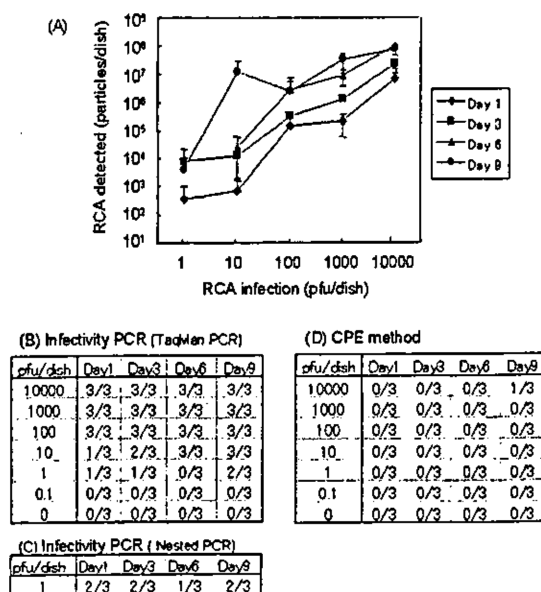
pfu/dish	Day1	Day3	Day6	Day9
10000	3/3	3/3	3/3	3/3
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	0/3	2/3	3/3	3/3
1	0/3	1/3	2/3	2/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(C) CPE method

pfu/dish	Day1	Day3	Day6	Day9
10000	0/3	0/3	3/3	3/3
1000	0/3	0/3	3/3	3/3
100	0/3	0/3	0/3	3/3
10	0/3	0/3	0/3	0/3
1	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

FIG. 5. Comparison of RCA detection by CPE assay and infectivity PCR. HeLa cells were infected with serial dilutions of RCA in medium. CPE was observed and cells were harvested on days 1, 3, 6, and 9. The viral DNA was extracted by glass beads and then the amount of RCA was determined by real-time quantitative PCR. (A) RCA growth curve in HeLa cells. Data are the means ± SD (n = 3). (B) The ratio of E1 DNA-positive samples is indicated (n = 3). (C) The number of CPE-positive samples is indicated (n = 3).

Copyright © The American Society of Gene Therapy



(B) Infectivity PCR (TaqMan PCR)

pfu/dish	Day1	Day3	Day6	Day9
10000	3/3	3/3	3/3	3/3
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	1/3	2/3	3/3	3/3
1	1/3	1/3	0/3	2/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(C) Infectivity PCR (Nested PCR)

pfu/dish	Day1	Day3	Day6	Day9
1	2/3	2/3	1/3	2/3

(D) CPE method

pfu/dish	Day1	Day3	Day6	Day9
10000	0/3	0/3	0/3	1/3
1000	0/3	0/3	0/3	0/3
100	0/3	0/3	0/3	0/3
10	0/3	0/3	0/3	0/3
1	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

FIG. 6. Comparison of the detection of RCA spiked into adenovirus vectors by CPE assay and infectivity PCR. HeLa cells were infected with serial dilutions of RCA spiked into 10^9 particles of adenovirus vectors. CPE was observed and cells were harvested on days 1, 3, 6, and 9. The viral DNA was extracted by glass beads, and then the amount of RCA was determined by real-time quantitative PCR or nested PCR. (A) RCA growth curve in HeLa cells. Data are the means ± SD ($n = 3$). (B and C) The ratio of E1 DNA-positive samples is indicated ($n = 3$). (D) The number of CPE-positive samples is indicated ($n = 3$).

Infectivity PCR for the Detection of RCA Spiked into Adenovirus Vector Products

Finally, we applied the infectivity PCR method for detecting RCA spiked into adenovirus vectors (Fig. 6). We infected 1.5×10^6 HeLa cells with 0, 0.1, 1, 10, 100, 1000, or 10,000 pfu of RCA spiked into 10^9 particles of adenovirus vectors (AdHM10LacZ-3). Because 10^9 particles of the adenovirus vector were equivalent to 5×10^7 infectious units, the multiplicity of infection (m.o.i.) was about 33 in this assay. We harvested the cells on days 1, 3, 6, and 9, and then we extracted the DNA using glass beads. By real-time quantitative PCR, we detected RCA in the samples that had been infected with more than 1 pfu of RCA (Figs. 6A and 6B). We detected no RCA in the samples infected with 0.1 pfu of RCA. Therefore we concluded the detection limit of this assay to be 1 pfu. This result was the same as that in the assay performed in the absence of adenovirus vectors. The absolute value of the amplified RCA was lower than in the assay without adenovirus vectors. When we also examined the presence of RCA by nested PCR (Fig. 6C), we detected E1 DNA in the samples infected with 1 pfu of RCA, but we did not detect it in those infected with 0.1 pfu of RCA, meaning that the detection limit was still 1 pfu. We observed a slight sign of CPE on day 9 in only one of the three dishes that had been infected with 10,000 pfu of RCA (Fig. 6D). CPE appeared to be suppressed by the presence of adenovirus vectors. From these results, when we measured RCA spiked into adenovirus vectors, the sensitivity of the assay was almost 10,000 times higher by infectivity PCR than by CPE assay. Thus the RCA contamination in adenovirus vectors could be detected with high sensitivity and short-term cell culture by the infectivity PCR method reported here.

Discussion

The infectivity PCR assay was developed for detecting RCA in adenovirus vectors (Fig. 7). The cell culture/CPE method in common use involves an infection of HeLa or A549 cells, harvesting, blind passage on fresh cells, and the search for CPE (Fig. 7A). It usually takes more than 4 weeks. In the method we reported here, HeLa cells are infected with adenovirus vector products and cultured for 3 or more days. The cells are harvested and viral DNA is extracted using glass beads, and then the amount of E1 DNA is measured by real-time quantitative PCR. At least 1 pfu of RCA spiked into adenovirus vectors can be detected by this method. The infectivity PCR method thus provides a more sensitive, rapid, and simple means of testing the quality of adenovirus vectors than the traditional CPE assay.

As a sensitive method for the detection of viral genome, PCR has been widely used. Real-time quantitative PCR is sensitive and highly reproducible over a wide dynamic range in addition to having high-throughput capacity. In our study, 10^3 to 10^8 particles of RCA could be detected quantitatively by real-time quantitative PCR. Nested PCR is known to be suitable for the detection of smaller amounts of DNA. By nested PCR, fewer than 10^1 particles of RCA could be detected. While the PCR method has advantages in terms of sensitivity, it has a disadvantage in that not only infectious virus but also uninfectious viral particles or DNA fragments are detected. In our experiments, we used primers and a probe for the E1 sequence that is present in RCA but not in adenovirus vectors. However, in addition to RCA, HEK293 cells in which the adenovirus vectors had been propagated had the E1 sequence in their genome. Although the adenovirus vectors had been purified by CsCl ultracentrifugation after treating the HEK293 cell lysate with nuclease, HEK293 cell-derived E1 DNA was detected in adenovirus vector products, and thus if the E1 DNA was detected in adenovirus vector products by PCR, it is not clear whether the origin is RCA or HEK293 cells. The presence of cellular E1 DNA in adenovirus vector products might not be limited to our sample, because purification by CsCl ultracentrifugation is the method used for the adenovirus vectors for clinical studies [20], and cellular DNA has been detected in adenovirus reference materials [21]. On the other hand, the fact that the ratio of infectious titer to particle concentration was 1:8.6 (see Materials and Methods) means that not all of the RCA particles

Copyright © The American Society of Gene Therapy

doi:10.1016/j.ymthe.2003.09.001

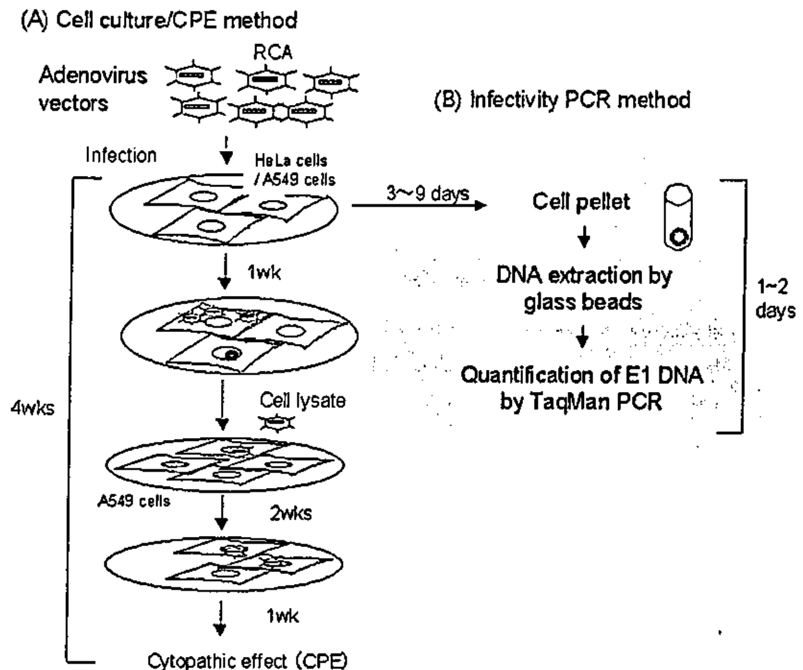


FIG. 7. (A) The cell culture/CPE method in common use involves an infection of HeLa or A549 cells, harvesting, blind passage on fresh cells, and the search for CPE. It usually takes more than four weeks. (B) In infectivity PCR method, HeLa cell are infected with adenovirus vector products, and cultured for three or more days. The cells are harvested and viral DNA is extracted using glass beads, and then the amount of E1 DNA is measured by real-time quantitative PCR.

were infectious. Therefore, infection of the adenovirus vectors into cells was thought to be indispensable for the selective amplification of the infectious RCA particles.

In the infectivity PCR assay, RCA could be detected with a detection limit of 1 pfu irrespective of the presence of adenovirus vectors. On the other hand, CPE was observed in the cells that had been infected with 10^2 to 10^4 pfu of RCA without adenovirus vectors or 10^4 pfu of RCA spiked into adenovirus vectors. Therefore, for the detection of RCA spiked into adenovirus vectors, the infectivity PCR was shown to be almost 10,000 times more sensitive than the CPE assay.

Since nested PCR was able to detect smaller amounts of DNA than real-time quantitative PCR, nested PCR was expected to contribute to an increase in the sensitivity of the infectivity PCR assay. However, the detection limit of the assay was still 1 pfu even when nested PCR was used. This might have been because, at lower concentrations of RCA, there was a threshold in the steps of infection or amplification in the cells, and thus the RCA was not linearly amplified.

The infectivity PCR was also shown to have the advantage of requiring fewer cells than the CPE assay. It is known that too high an input m.o.i. may lead to suppression of RCA outgrowth by the vector [12,22]. Because adenovirus vectors are prepared as high-titer stocks, a large-scale cell culturing, e.g., roller-bottle culture, is required to test the presence of RCA at a low m.o.i. Although the presence of adenovirus vectors seemed to interfere with the CPE, RCA could be detected by real-time quantitative PCR with the same detection limit of 1 pfu even in the presence of adenovirus vectors. This means that infectivity PCR can be performed at a higher m.o.i. than the CPE assay and therefore may contribute to a decrease in the number of the cells required for the assay.

The FDA currently recommends that adenovirus vector preparations contain <1 RCA in 3×10^{10} particles. Since, in our method, 10^9 particles of adenovirus vectors can be tested using one 10-cm-diameter plate, 3×10^{10} particles of adenovirus vectors can be tested using 30 plates. If 3×10^{10} particles are tested in the standard culture method with blind passage and come out positive, the only possible conclusion would be that there was >1 pfu in 3×10^{10} particles. Our method has the potential to be much more effective and quantitative than the standard culture method, because each plate can be tested separately, and then the actual level of RCA contamination can be estimated using the Poisson distribution.

With a goal of ensuring the safety of gene therapy, official guidelines have been published for the testing of replication-competent retroviruses (RCR) [23]. These guidelines, entitled *Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-Up of Patients in Clinical Trials Using Retroviral Vectors,* provide information on RCR testing during manufacture (including timing), amount of material to be tested, and general testing methods. However, with respect to RCA in adenovirus vectors, the only available guidelines are the descriptions included within the *Guidance for Human Somatic Cell Therapy and Gene Therapy* [12]. In these guidelines, the cell culture/

Copyright © The American Society of Gene Therapy

CPE method is recommended for testing the presence of RCA. Although it is recommended that the assay sensitivity should be validated by spiking the test inocula with increasingly smaller numbers of wild-type adenovirus particles, there are currently no detailed guidelines for the RCA test. With regard to the safety concerns of adenovirus vector-based gene therapy, most research has focused on the immune response against capsid proteins [24]. In addition, preclinical safety studies are inherently limited in their assessment of RCA-related risks, since there are no animal models that support extensive replication of human wild-type adenovirus. This fact may have limited the attention paid to establishing an RCA detection method. Our method reported here could be used to test the RCA contamination in clinical lots of adenovirus vectors or to detect RCA in patients undergoing adenovirus vector-based gene therapy. Together with the recent efforts to develop cell lines that can propagate adenovirus vectors without emergence of RCA [25–27], this method might improve the safety of adenovirus vector-based gene therapy.

Materials and Methods

Cells. HeLa cells were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan) and maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). HEK293 cells were obtained from Clontech (Palo Alto, CA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

Replication-competent adenovirus. The seed RCA (adenovirus type 5) was purchased from ATCC (Manassas, VA). For the amplification of the RCA, HeLa cells were infected with the RCA and harvested when CPE was observed. The cell lysate was prepared by four cycles of freezing and thawing and then was added to another dish of HeLa cells. After the amplification was repeated, the cell lysate from five 150-mm-diameter dishes was subjected to RCA purification by CsCl ultracentrifugation. The particle concentration and infectious titer of RCA were measured according to the standard operating procedure for adenovirus reference material (ATCC VR1516). For determining the particle concentration, RCA was diluted with the excipient solution (20 mM Tris, 25 mM NaCl, 2.5% glycerol (w/v), pH 8.0). After incubation for 15 min at room temperature, the absorbance value at 260 nm was measured. The particle concentration was determined as 5.826×10^{11} particles/ml. For determining the infectious titer, HEK293 cells were seeded in 96-well plates at 4×10^4 cells/well. After culturing for 1 day, medium was replaced with 200 μl of medium containing 5×10^7 to 1.28×10^{10} times diluted RCA stock. The plates were incubated for 60 min, and then the medium was replaced with 200 μl of fresh medium. On day 10, each well was examined for signs of CPE using a light microscope. The infectious titer was calculated as the normalized adjusted standard titer [28] and determined to be 6.767×10^{10} NIU/ml. The ratio of infectious titer to particle concentration was 1:8.6.

After we had prepared our in-house RCA standard, the Adenovirus Reference Material (ATCC VR-1516) developed under the guidance of the Adenovirus Reference Material Working Group and the U.S. Food and Drug Administration was distributed [21]. The ratio of infectious titer to particle concentration of the reference material is 1:8.3. The ratio of our in-house RCA was 1:8.9 (5.8×10^{11} particles/ml vs 6.5×10^{10} IU/ml) when these values were measured using the Adenovirus Reference Material as a reference. Since the ratios for our in-house standard and the reference material were similar, our in-house RCA standard was considered to have qualities similar to those of the reference material.

Adenovirus vector. Adenovirus vectors were prepared as described previously [29]. In brief, the plasmid harboring β-galactosidase in the E1-deleted region of the adenovirus, pAdHM10LacZ, was digested with *PacI*. The linearized plasmid was transfected into subconfluent HEK293 cells plated in a 60-mm dish using SuperFect (Qiagen, Valencia, CA) according to the manufacturer's protocol. Ten days later, cells were harvested and adenovirus vectors were released by four cycles of freezing and thawing. The vectors were amplified by further infecting into HEK293 cells and then purified by CsCl step gradient ultracentrifugation followed by CsCl linear gradient ultracentrifugation.

Real-time quantitative PCR. The ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) was used for detecting real-time quantitative PCR products. The DNA extracted from RCA standard or RCA-infected cells was dissolved in 20 μl of distilled H_2O. Ten microliters of the DNA sample was used as template in a subsequent real-time quantitative PCR with 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems). The PCR was initially denatured at 95°C for 10 min and then subjected to cycles of 95°C for 15 s and 60°C for 1 min. The reaction was carried out for 50 cycles. A calibration curve was generated for the DNA extracted from purified RCA and validated using linear regression analysis. The sequences of the primers and probe used were as follows: Ad5dE1-1035F, TCCGGTCCTTCTAACACACCTC; Ad5dE1-1105R, ACGGCAACTGGTTTAATGGG; and Ad5dE1-1058TM probe, FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA. These sequences were designed using Primer Express software version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired molecular weight.

Nested PCR for E1 DNA. The primers used for the first PCR were Jzp5-Ad3473F, CGCTGAGTTTGGCTCTAGCGAT, and Jzp6-Ad3698R, CATCACATTCTGACGCACCC. The primers for the second PCR were Jzp5-2-Ad3483F, GGCTCTAGCGATGAAGATACAG, and Jzp6-2-Ad3668R, GGGCATGCGCGTTGTCAAAT. The amplification conditions for the PCR consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. Two microliters of the 50-μl reaction mixture from the first PCR was subjected to a second PCR. For the second PCR, the number of amplifications was set to 25.

PCR for pregnancy-specific glycoprotein gene. To detect the DNA derived from HEK293 cells, a pregnancy-specific glycoprotein gene adjacent to the E1 gene in HEK293 cells was selected as a target. The primers used for the PCR were PSG3-293-5281F, CTCATGCCTGCCTCTTTCACT, and PSG4-293-5567R, AGAGCCATCCA CACAATGTGC.

Nucleic acid extraction using SMI TEST EX-R&D. Cells were harvested and centrifuged at 2000 rpm for 5 min and then suspended in PBS(−). After four cycles of freezing and thawing, the nucleic acids derived from the cells were digested by incubating with DNase I (0.2 mg/ml), RNase A (0.2 mg/ml), and $MgCl_2$ (10 mM) at 37°C for 30 min. Then DNA was extracted using SMI TEST EX-R&D (Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. When DNA was extracted from purified RCA or adenovirus vectors, the samples were subjected directly to DNA extraction using SMI TEST EX-R&D reagents.

Nucleic acid extraction using glass beads. Glass beads (GSB 07) approximately 7 mm in diameter were obtained from Nippon Rikagaku Kikai Co. (Tokyo, Japan). The glass beads were treated with 30% hydrogen fluoride (HF) solution for 1 h and then extensively washed with distilled water. The HF-treated glass beads were used for the extraction of viral genome.

For nucleic acid extraction, cells were harvested in 10-ml tubes and centrifuged at 2000 rpm for 5 min. After the medium was removed, one glass bead and 250 μl of extraction solution (200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1% *N*-lauroylsarcosinate, 1% SDS, 2% 2-mercaptoethanol, and 1 mg/ml proteinase K) were added to each tube, and then the samples were stirred for 10 s. After incubation at 65°C for 20 min, 2 ml of 70% isopropyl alcohol was added. The samples were incubated at room temperature for 1 min with gentle stirring. The solution was removed, and the beads were washed with 2 ml of 75% ethanol twice. Then the beads were

Copyright © The American Society of Gene Therapy

doi:10.1016/j.ymthe.2003.09.001

dried at room temperature. DNA was eluted from each bead by adding 50 μl of distilled H_2O and incubating at 65°C for 5 min. Twenty microliters of the sample was assayed for E1 DNA.

Infectivity PCR using HeLa cells. HeLa cells (1.5×10^6) were seeded in 100-mm-diameter dishes. One day later (day 0), the cells were infected with 1 to 10^4 pfu of RCA spiked into 10^9 particles of adenovirus vectors by incubating at 37°C for 2 h in 1 ml of MEM containing 1% FCS. After infection, the medium was replaced with MEM containing 10% FCS. On days 1, 3, 6, and 9, the cells and supernatant were harvested. DNA was extracted, and the amount of E1 DNA was measured by real-time quantitative PCR. The experiments were done in triplicate.

Acknowledgments

This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan and by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science.

RECEIVED FOR PUBLICATION JULY 15, 2003; ACCEPTED SEPTEMBER 4, 2003.

References

1. http://www.wiley.co.uk/wileychi/genmed/clinical/.
2. Mizuguchi, H., *et al.* (2001). A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther.* 8: 730–735.
3. Mizuguchi, H., *et al.* (2002). CAR- or αv integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. *Gene Ther.* 9: 769–776.
4. Belousova, N., Krendelchtchikova, V., Curiel, D. T., and Krasnykh, V. (2002). Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. *J. Virol.* 76: 8621–8631.
5. Koizumi, N., *et al.* (2001). Efficient gene transfer by fiber-mutant adenoviral vectors containing RGD peptide. *Biochim. Biophys. Acta* 1568: 13–20.
6. Mizuguchi, H., and Hayakawa, T. (2002). Enhanced antitumor effect and reduced vector dissemination with fiber-modified adenovirus vectors expressing herpes simplex virus thymidine kinase. *Cancer Gene Ther.* 9: 236–242.
7. Mizuguchi, H., and Hayakawa, T. (2002). Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. *Gene* 285: 69–77.
8. Mizuguchi, H., and Hayakawa, T. (2001). Characteristics of adenovirus-mediated tetracycline-controllable expression system. *Biochim. Biophys. Acta* 1568: 21–29.
9. Mizuguchi, H., and Hayakawa, T. (2002). The tet-off system is more effective than the tet-on system for regulating transgene expression in a single adenovirus vector. *J. Gene Med.* 4: 240–247.
10. Sakhuja, K., *et al.* (2003). Optimization of the generation and propagation of gutless adenoviral vectors. *Hum. Gene Ther.* 14: 243–254.
11. Lochmuller, H., *et al.* (1994). Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (Δ E1 + Δ E3) during multiple passages in 293 cells. *Hum. Gene Ther.* 5: 1485–1491.
12. Guidance for human somatic cell therapy and gene therapy (2001). *Hum. Gene Ther.* 12: 303–314.
13. Roitsch, C., *et al.* (2001). Characterization and quality control of recombinant adenovirus vectors for gene therapy. *J. Chromatogr. B Biomed. Sci. Appl.* 752: 263–280.
14. Zhu, J., *et al.* (1999). Characterization of replication-competent adenovirus isolates from large-scale production of a recombinant adenoviral vector. *Hum. Gene Ther.* 10: 113–121.
15. Zhang, W. W., Koch, P. E., and Roth, J. A. (1995). Detection of wild-type contamination in a recombinant adenoviral preparation by PCR. *Biotechniques* 18: 444–447.
16. Simmonds, P., *et al.* (1990). Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J. Virol.* 64: 864–872.
17. Yei, S., Yu, M. W., and Tankersley, D. L. (1992). Partitioning of hepatitis C virus during Cohn–Oncley fractionation of plasma. *Transfusion* 32: 824–828.
18. Louis, N., Evelegh, C., and Graham, F. L. (1997). Cloning and sequencing of the cellular–viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology* 233: 423–429.
19. Aritomi, T., *et al.* (1998). Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis. *Jpn. J. Gastroenterol. Hepatol.* 11: 1125–1132.
20. Harvey, B. G., *et al.* (1999). Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. *J. Virol.* 73: 6729–6742.
21. Hutchins, B. (2002). Development of a reference material for characterizing adenovirus vectors. *BioProcessing J.* 1: 25–28.
22. Dion, L. D., Fang, J., and Garver, R. I. Jr. (1996). Supernatant rescue assay vs. polymerase chain reaction for detection of wild type adenovirus-contaminating recombinant adenovirus stocks. *J. Virol. Methods* 56: 99–107.
23. Supplemental guidance on testing for replication-competent retrovirus in retroviral vector-based gene therapy products and during follow-up of patients in clinical trials using retroviral vectors (2001). *Hum. Gene Ther.* 12: 315–320.
24. Assessment of adenoviral vector safety and toxicity: report of the National Institutes of Health Recombinant DNA Advisory Committee (2002). *Hum. Gene Ther.* 13: 3–13.
25. Gao, G. P., Engdahl, R. K., and Wilson, J. M. (2000). A cell line for high-yield production of E1-deleted adenovirus vectors without the emergence of replication-competent virus. *Hum. Gene Ther.* 11: 213–219.
26. Fallaux, F. J., *et al.* (1998). New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum. Gene Ther.* 9: 1909–1917.
27. Schiedner, G., Hertel, S., and Kochanek, S. (2000). Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum. Gene Ther.* 11: 2105–2116.
28. Nyberg-Hoffman, C., Shabram, P., Li, W., Giroux, D., and Aguilar-Cordova, E. (1997). Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat. Med.* 3: 808–811.
29. Mizuguchi, H., and Kay, M. A. (1998). Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum. Gene Ther.* 9: 2577–2583.

Copyright © The American Society of Gene Therapy

Virus Concentration Using Sulfonated Magnetic Beads to Improve Sensitivity in Nucleic Acid Amplification Tests

Akiko IWATA,[a] Koei SATOH,[b] Mitsuhiro MURATA,[c] Mikio HIKATA,[c] Takao HAYAKAWA,[a] and Teruhide YAMAGUCHI*,[a]

[a] The National Institute of Health Sciences; 1-18-1 Kamiyoga, Tokyo 158-8501, Japan: [b] The Institute of Saitama Red Cross Center; 8-3-41 Kamiochiai, Saitama, Saitama 338-0001, Japan: and [c] JSR Corporation, Tsukuba Research Laboratories; 25 Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan. Received February 28, 2003; accepted April 26, 2003

To enhance the sensitivity of virus detection by polymerase chain reaction (PCR) and reverse-transcriptional (RT)-PCR, we developed a novel virus-concentration method using sulfonated (SO-) magnetic beads in the presence of divalent cations. In the presence of either Zn^{2+} or Cu^{2+} ions, we showed that SO-magnetic beads were able to concentrate non-enveloped model viruses, such as porcine parvovirus (PPV) and poliovirus, which were not concentrated by polyethyleneimine (PEI)-magnetic beads.[1)] Using the SO-magnetic beads, the sensitivity of virus genome detection by PCR or RT-PCR can be enhanced. Therefore, an efficient virus concentration method using either SO-magnetic beads or PEI-magnetic beads enhances the sensitivity of virus detection by PCR or RT-PCR.

Key words polymerase chain reaction (PCR); virus; divalent cation; nucleotide amplification tests (NAT)

Many useful biological drugs (biologicals) are produced from blood or by cell-culture techniques. Furthermore, new biologicals such as gene-therapy and cell-therapy products have created new hope for the treatment of grave genetic diseases or lethal ailments. These conventional and innovative biologicals, however, involve some risks regarding the spread of transmissible diseases such as virus-mediated infectious diseases. To ensure the viral safety of these biologicals, viral tests should be performed at the various stages of the drug production process, namely the raw material, cell bank, intermediate product, and final product stages. The development of more sensitive methods of virus detection is pivotal for the viral safety of new biotechnology products, especially for cell therapy or gene therapy, because in these new therapeutic technologies the target cells or cell-derived vectors are directly induced in patients.

Polymerase chain reaction (PCR) is a highly sensitive method for the detection of virus genomes.[2)] In many countries, recently, several nucleotide amplification tests (NAT) including PCR have been employed to screen for specific viruses in raw materials such as blood products and cell therapy products.[3)] These tests are able to detect several copies of virus genome,[4—7)] but there is a detection limit even in the NAT methods. For example, it has been reported that these methods are unable to detect the viral genome in the early stages of specific viral infections. Therefore, if target viruses could be concentrated, the sensitivity of NAT must be enhanced.

Several approaches to the concentration of viruses have been attempted to enhance the sensitivity of virus genome detection.[8—10)] Ultracentrifugation of viruses has been shown to be a superior method of virus concentration, but it is very time-consuming and not suitable for screening. Polyethyleneglycol (PEG) precipitation techniques are simple and make it easy to concentrate viruses, but the excess amount of PEG in the extract hampers the PCR reaction. Therefore, a simple and feasible method for virus concentration is needed.

Recently, we reported that polyetheleimine-conjugated magnetic (PEI-magnetic) beads efficiently concentrated the enveloped model viruses and simianvirus 40 (SV-40).[1)] However, PEI-magnetic beads could not be applied to some nonenveloped viruses, such as poliovirus or porcine parvovirus (PPV).

In the present study, we report that the sulfonated-magnetic (SO-magnetic) beads were useful for the concentration of poliovirus or PPV in the presence of divalent cations, such as Zn^{2+} or Cu^{2+} ions. We discuss herein the usefulness of SO-magnetic beads in enhancing the sensitivity of virus genome detection by PCR or RT-PCR.

MATERIALS AND METHODS

Materials Magnetic beads, IMMUTEX-MAG™ (JSR Inc., Japan), were used throughout this study. IMMUTEX-MAG beads are acrylic latex beads that contain magnetite inside and are conjugated with carboxylic groups on the surface. The IMMUTEX-MAG beads used in the present study were 0.8 μm in diameter, and the surface charge density was 21 μmol/g. Polyethyleneimine (PEI, Mw 70000) and 1-ethylene-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) were purchased from Wako Pure Chemical Inc., Japan. The primer sets used in the PCR or RT-PCR for the detection of viruses were used as reported previously[11,12)] or designed from the virus genomic information (Table 1).

Table 1. Primer Sets Used in the PCR and RT-PCR

	Primer set
Sindbis virus	5'-GGATTGGSTTYGAYACCACYCAGTTCATGT-3'
	5'-TGCCCYATGCGKAGYCCMGAAGAYCC-3'
PPV[a)]	5'-TTGGTAATGTTGGTTGCTACAATGC-3'
	5'-TATGTTCTGGTCTTTCCTCGCATC-3'
Poliovirus[b)]	5'-TGCGAGATACCACACAT-3'
	5'-ATCATGCTTTCAAGCATCTG-3'
Cytomegalovirus	5'-TGCCGGATGATGCAGGA-3'
	5'-GTGAGCCCGACGGGTTCT-3'

a, b) Each primer set was prepared according to the original papers: PPV[11)] and Poliovirus.[12)] The primer sets of Sindbis virus and cytomegalovirus were designed according to the sequence published.

* To whom correspondence should be addressed. e-mail: yamaguch@nihs.go.jp

© 2003 Pharmaceutical Society of Japan

Viruses In the present study, we used the model viruses that have been used for viral clearance study as recommended in the international harmonized guidelines.[13] Sindbis virus and porcine parvovirus (strain 90HS) (PPV) were kindly donated by Dr. Kohase (National Institute of Infectious Diseases). Poliovirus (strain Sabin) was kindly donated by Dr. Koga (JCR Co., Japan). The supernatants of Vero cells infected with either poliovirus or Sindbis virus were used as the virus samples. The supernatant of ESK cells infected with porcine parvovirus was used as the PPV sample. In order to remove the cell-debris from the collected virus suspension, each virus suspension was centrifuged at 3000 rpm for 10 min. After removing the celldebris, the resulting stock viruses were aliquoted and stored at −80 °C until use. The monkey cytomegalovirus (ATCC VR-677) derived from the supernatant of MRC-5 cells infected by the virus was obtained from ATCC.

Sulfonated Magnetic Beads Sulfonated-magnetic beads were prepared as follows; 10 g of IMMUTEX-Mag (JSR Corp. Tokyo, Japan) were dispersed in 100 g of water containing 0.2 g of sodium dodecylsulfate. To the dispersion obtained, 3 g of methyl methacrylate and 3 g of 2-acrylamide-2-methylpropane sulfonic acid sodium salt were added, and the mixture was heated to 80 °C, and thereafter 0.2 g of perbutyl-O (NOF Corp. Tokyo, Japan) was added to carry out seed-polymerization for 5 h. After the reaction was completed, the beads were washed with distilled water and then with phosphate-buffered saline (PBS) to obtain SO-magnetic beads.

Viral Concentration Using Sulfonate-Magnetic Beads The essential adsorption procedure for each virus was as follows; 1 or 10 ml of each virus supernatant was incubated both with various concentrations of divalent cations and 100 μl SO-magnetic beads for 10 min at room temperature, and then the complex of virus and magnetic beads was trapped by a magnetic field (Magnetic Trapper™, Toyobo Co., Japan; Dynal MOC-1™, Dynal AS., Oslo), after which the medium was aspirated. The trapped virus and bead complexes were resuspended in an aliquot of medium to be adjusted to 100 μl, and then the extraction of virus RNA or DNA was carried out from the 100 μl of virus-bead complexes or the virus solution with a SMI-TEST EX-R&D kit (Genome Science Laboratories, Fukushima, Japan). The extracted RNA and DNA were dissolved in 20 μl of TE buffer (10 mM Tris–HCl, pH 7.4/0.1 mM EDTA).

Determination of Viral-Infectivity The infectious titer of each virus suspension was determined using each indicator cell. Sindbis virus and poliovirus were introduced into Vero cells, and PPV was introduced into ESK cells.[14—16] The virus suspension and PEI-magnetic bead-treated suspension were subjected to infectious assay ($TCID_{50}$ assay) using each indicator cell. Cells were maintained as stock cultures in α-MEM and replated 2 d before infection in 96-well plates for the $TCID_{50}$ assay.[17] Each virus suspension was serially diluted. Cell cultures were then infected for 1 h. The medium for dilution and infections was Hank's balanced solution supplemented with 20 mM HEPES buffer (pH 7.4). Postinfection $TCID_{50}$ cultures were washed and then fed with the media. After 48 or 72 h of incubation postinfection, the MTT assay was carried out after the degeneration of virus-infected cells became evident.[18] The MTT method utilizes 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT), which is converted to a water-insoluble blue crystal (formazan) in viable cells through mitochondrial dehydrogenase activity.[19] Dead cells are unable to reduce MTT to formazan; thus, the amount of formazan present is an indication of the number of viable cells. The optical density (OD) of formazan was determined using a computer-controlled microplate reader.

Table 2. Primer and Probe Sets Used in the Real-Time PCR and Real-Time RT-PCR

	Primer and probe set
PPV	5′-CGTGGAGCGAGCCAACA-3′
	5′-AGTTGGGACTTGGTGTCCGTATTG-3′
	FAM-labeled 5′-TGCACTTAACTCCAACACCGCCAGATT-3′
Poliovirus	5′-ATTACACGCTGACACAAAACCAAG-3′
	5′-ATAGTTTCACCGAAGGCGGA-3′
	FAM-labeled 5′-CTAGAATAGTCGTCCTCCCTCTTTCGA-CACCCAG-3′

DNA and RNA from Sindbis virus, VSV and SV-40 virus were quantitatively determined using Syber Green PCR Master Mix™ (Applied Biosystems).

Viral Genome Amplification The PCR was carried out in a 50-μl reaction mixture containing 25 μl of PCR-Master Mix Reagent Kit (Applied Biosystems Co.), 13 μl of DNase-free distilled water, 0.5 μM of each primer set, and 10 μl of extracted sample. PCR was performed on a GeneAmpPCR System 9700 (Applied Biosystems Co.). Thirty-five cycles at 95 °C for 30 s (5 min during cycle 1), annealing at 55 °C for 30 s, and extension at 72 °C were carried out. After the final cycle, the tubes were incubated for an additional 5 min at 72 °C and cooled down to 5 °C. The reverse-transcriptional PCR was carried out in a 50-μl reaction mixture containing 25 μl of RT-PCR-Master Mix Reagent Kit (Applied Biosystems Co.), 13 μl of DNase/RNase-free distilled water, 0.5 μM of each primer set, and 10 μl of extracted sample. RNA virus genome was reverse-transcribed into cDNA (45 min at 50 °C), and the target fragments were amplified by PCR, with 35 cycles at 95 °C for 30 s (5 min during cycle 1), annealing at 55 °C for 30 s, and extension at 72 °C. After amplification, electrophoretic separation of the PCR products (10 μl) was performed on 2% agarose gels in 1x Tris-borate-EDTA buffer (pH 8.4), and the PCR products were stained with ethidium bromide and visualized with UV illumination.

Real-Time PCR on the ABI PRISM 7000 Real-time PCR was carried out in a 50-μl reaction mixture containing 25 μl of TaqMan Gold Master Mix (Applied Biosystems Co.) or 25 μl of Syber Green PCR Master Mix (Applied Biosystems Co.), 13 μl of DNase/RNase-free distilled water, 0.5 μM of each primer set with or without a fluorescence probe, and 10 μl of extracted sample. The reaction mixture for PCR was prepared in a single tube as follows; at first, the RNA virus genome was reverse-transcribed into cDNA (50 min at 42 °C), and the reaction was terminated for 15 min at 95 °C. The reaction mixture was then mixed with 25 μl of Syber Green RT-PCR Master Mix, 13 μl of DNase/RNase-free distilled water, and 0.5 μM of each primer set. The real-time PCR was monitored on the ABI PRISM 7000 Sequence-Detection System (Applied Biosystems Co.).

RESULTS

Since several reports suggested that divalent cations interact with viruses,[20—24] we expected the viruses to form complexes with divalent cations. We tried to trap the complexes of virus and divalent cation with negatively charged SO-magnetic beads. To clarify whether the model viruses interacted with the SO-magnetic beads in the presence of divalent cations, we compared the infectious titers of virus suspension treated with SO-magnetic beads to the untreated suspension. As shown in Table 3, in the presence of 10 mM Zn^{2+} ion, the infectious titers of Sindbis virus, PPV, and poliovirus suspension treated with SO-magnetic beads decreased dramatically compared to those of the untreated samples. The infectious titers of Herpes Simplex I, vesicular stomatitis virus, and SV-40 virus were also decreased by the treatment with SO-magnetic beads (data not shown).

We next analyzed the virus-genome content of SO-magnetic bead-adsorbed fractions by PCR or RT-PCR. Figure 1 shows that in the presence of 10 mM Zn^{2+} ion, the treatment with SO-magnetic beads concentrated the Sindbis virus, PPV and poliovirus genome as compared with untreated fractions. Similar results were obtained in the presence of 10 mM Cu^{2+} ion instead of Zn^{2+} ion. These results suggest that in the presence of divalent cations such as Zn^{2+} ion or Cu^{2+} ion, SO-magnetic beads can adsorb PPV and poliovirus which could not be adsorbed by PEI-magnetic beads*.

Table 3. Reduction of Infectivities in Viruses Treated with SO-Magnetic Beads in the Presence of Zn^{2+} Ions

		Concentration of infectious virus (×log 10)
Sindbis virus	Applied sample	6.5±4.8[a)]
	Upper layer treated with SO-beads	4.3±2.0
PPV	Applied sample	6.8±5.3
	Upper layer treated with SO-beads	4.5±2.4
Poliovirus	Applied sample	7.0±5.7
	Upper layer treated with SO-beads	3.3±1.5

a) Means±S.D. (*n*=4).

Next, to examine the effects of divalent cations on the adsorption efficiency of SO-magnetic beads, the virus genome in the concentrated samples was quantitatively determined by real-time PCR. As shown in Fig. 2, the PPV-trapping efficiency by SO-magnetic beads was most effective in the presence of either 5—10 mM Zn^{2+} or Cu^{2+} ions. In the case of poliovirus, the most effective concentration of poliovirus was observed in the presence of 10 mM Cu^{2+} ion. On the other hand, in the presence of Mn^{2+} ion, SO magnetic beads only weakly adsorbed either poliovirus or PPV. In the presence of Ca^{2+} ion or Mg^{2+} ion the effective virus adsorption could not be observed (data not shown). Concerning the effect of divalent ions, the same tendency was observed for the adsorption of Sindbis virus or SV-40 virus (data not shown). The following experiment was performed in the presence of either 10 mM Cu^{2+} ion or Zn^{2+} ion.

Next, 1 or 10 ml of serially diluted suspension of poliovirus and PPV was concentrated to 100 μl with SO-magnetic beads in the presence of 10 mM Cu^{2+} ion. After the adsorption to SO-magnetic beads, the virus genome in the complex of virus and SO-magnetic beads was extracted, and then the copy numbers were compared to that in the original suspension by means of quantitative PCR and RT-PCR. As shown in Fig. 3, poliovirus and PPV, which were diluted to various concentrations with the medium, were efficiently concentrated by SO-magnetic beads. While the 10-fold concentration procedures for poliovirus and PPV by SO-magnetic beads resulted in the expected concentrations from the original solutions, respectively, in the case of 100-fold concentration, sufficient recovery was not observed for poliovirus or PPV. On the other hand, the detection limit of poliovirus and PPV could be lowered by the concentration using the SO-magnetic bead method. The same results were obtained for Sindbis virus or SV-40 (data not shown).

Finally, we examined the absorption ability of SO-magnetic beads and PEI-magnetic beads for human disease virus. As shown in Fig. 4, both magnetic beads efficiently absorbed cytomegalovirus. This result also clearly demonstrated that both magnetic beads are useful for the concentration of viruses.

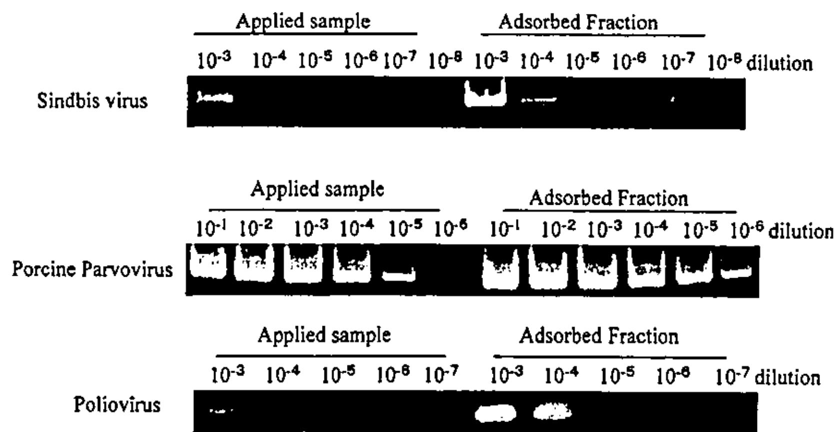


Fig. 1. Concentration of Model Viruses by SO-Magnetic Beads

One milliliter of virus suspension was incubated with 100 μl of SO-magnetic beads. The virus genomes in the complex of viruses and SO-magnetic beads were extracted as described in "Materials and Methods." The virus genomes in the serial dilutions of virus suspensions were compared with those in the SO-magnetic bead-concentrated samples by PCR or RT-PCR.

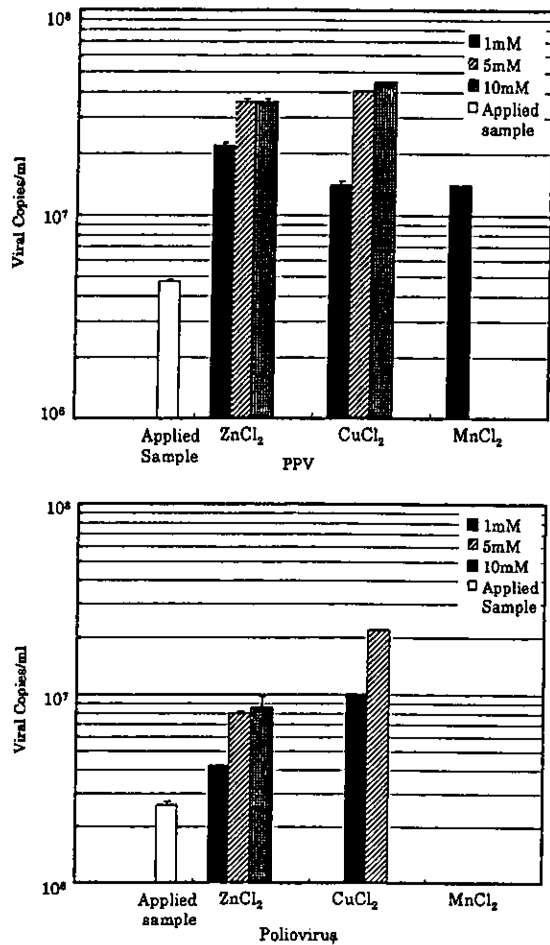


Fig. 2. Concentration of PPV and Poliovirus by SO-Magnetic Beads in the Presence of Various Amounts of Divalent Cations

One milliliter of PPV or poliovirus suspension was incubated with SO-magnetic beads in the presence of various amounts of Cu^{2+}, Zn^{2+} or Mn^{2+} ions. After the incubation, viruses bound to SO-magnetic beads were resuspended in 100 μl of medium, and the virus genome was then extracted as described in "Materials and Methods." The copy numbers of virus genome (original) in the serial dilutions of virus-infected medium were determined by real-time PCR or RT-PCR.

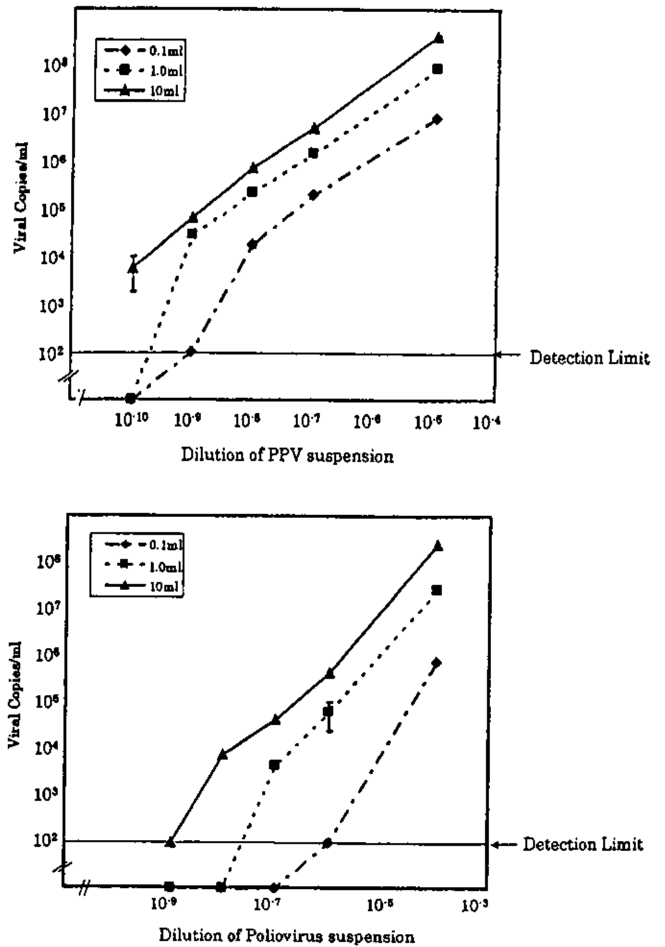


Fig. 3. Quantitative Analysis of Virus Concentration by SO-Magnetic Beads

One milliliter (×10) or 10 ml (×100) of virus suspension was incubated with SO-magnetic beads. After the incubation, the viruses bound to SO-magnetic beads were resuspended in 100 μl of medium, and the virus genome was then extracted as described in "Materials and Methods." The copy numbers of the virus genome (0.1 ml) in the serial dilutions of virus-infected medium were compared with those in the extract (1.0 ml and 10 ml) of SO-magnetic beads by real-time PCR or RT-PCR. The vertical axis indicates the copy numbers of viral genome in the extract.

DISCUSSION

Previously, we reported that PEI-magnetic beads efficiently adsorbed many viruses, such as Sindbis virus, SV-40, HSV-1 or VSV, resulting in the enhancement of the sensitivity of virus detection by PCR or RT-PCR*[1]. Some nonenveloped viruses, such as PPV or poliovirus, however, could not be adsorbed by PEI-magnetic beads. We, therefore, tried to establish a new method for the concentration of PPV or poliovirus. Divalent cations, such as Cu^{2+} or Zn^{2+} ions, have been reported to interact with several species of viruses.[20—24] We, therefore, suspected that after incubation with viruses and divalent cations, the SO-magnetic beads may adsorb the complex of virus and divalent cations. The present study shows that the SO-magnetic beads adsorb many viruses, including not only Sindbis virus or SV-40, but also PPV or poliovirus. In particular, the most effective concentration of poliovirus or PPV by SO-magnetic beads was observed in the presence of either Zn^{2+} ion or Cu^{2+} ion, compared with Mn^{2+}, Ca^{2+} or Mg^{2+} ions. In the absence of divalent cations, the SO-magnetic beads did not adsorb any viruses (data not shown). The analysis by PCR or RT-PCR of virus genome

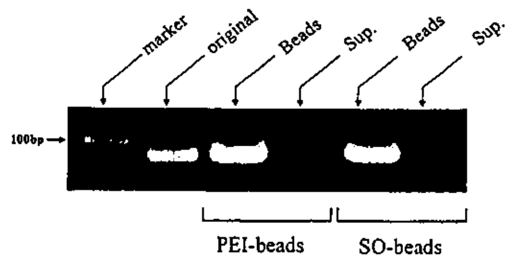


Fig. 4. Concentration of Cytomegalovirus by SO-Magnetic Beads or PEI-Magnetic Beads

One milliliter of cytomegalovirus suspension was mixed and then incubated with either SO-magnetic beads or PEI-magnetic beads. After the incubation, the viruses bound to both magnetic beads were resuspended in 100 μl of medium, and the virus genome from magnetic beads fractions (Beads) and supernatant fractions (Sup.) was then extracted as described in Materials and Methods. The virus genome in the extract was amplified by PCR.

copies in the concentrated fraction derived from the serial dilution of viruses by SO-magnetic beads reveals that SO-magnetic beads can be used to improve the sensitivity of the detection limit of virus genomes by PCR or RT-PCR. Whereas sufficient recovery of the virus genome to 100 μl did not

occur with 10 ml virus suspension, quantitative recovery to 100 μl was observed with the 1 ml virus suspension. The concentration of CMV obtained using PEI-magnetic beads was compared with that obtained using SO-magnetic beads, and it was found the efficiencies of both methods were comparable. The most significant point is that the virus concentration by SO-magnetic beads is applicable to PPV and poliovirus, which is not adsorbed by PEI-magnetic beads.

The virus-adsorption mechanism of SO-magnetic beads remains unclear at present. Both Zn^{2+} and Cu^{2+} ions were reported to inactivate or interfere with many viruses.[22—24] Kümel *et al.*[22] showed that Zn^{2+}-inactivated virus cannot properly penetrate a susceptible cell because the bound Zn^{2+} ion functionally inactivated the viral surface glycoprotein. Electron micrographs showed deposition of zinc onto the virion surface, which blocked presentation to the cell of a virion binding site.[22] Chruscinski *et al.*[20] reported the specific interaction of Cu^{2+} ions with fragments of envelope protein of hepatitis B virus. These data indicate that some divalent cations interact with virus surface protein or phospholipid. In these contexts, we suppose that some divalent cations form a complex with viruses, and the resulting complex of divalent cation and virus may be adsorbed by SO-magnetic beads that are negatively charged. In the present study, we successfully concentrated some nonenveloped viruses that could not be concentrated by PEI-magnetic beads as reported previously. Divalent cations are also suspected to interact with the surface phospholipids of enveloped viruses.

In conclusion, in the presence of divalent cations such as Cu^{2+} ions or Zn^{2+} ion, SO-magnetic beads efficiently concentrated many viruses, resulting in an increment in the sensitivity of the detection of the virus genomes.

Acknowledgement This work was supported in part by a grand-in-aid for health science research (H12-SAISEI-019) from the Japanese Ministry of Health, Labor and Welfare.

REFERENCES

1) Satoh K., Iwata A., Murata M., Hikata M., Hayakawa T., Yamaguchi T., *J. Virol. Methods*, under submission.
2) Saiki R. K., Stoffel S., Scharf S. J., Higuchi R., Horn G. T., Mullis K. B., Erlich H. A., *Science*, **239**, 487—491 (1988).
3) Willkommen H., Schmidt I., Lower J., *Biologicals*, **27**, 325—331 (1999).
4) Sarrazin C., Kokka R., Rabenau H., Zeuzem S., *Hepatology*, **32**, 818—823 (2000).
5) Kamisango K., Sumi M., Goto S., Hirao A., Gonzales F., Yasuda K., Iino S., *J. Clin. Microbiol.*, **3**, 310—314 (1999).
6) Alter H. J., Sanchez-Pescandor R., Urdea M. S., Wilber J. C., Lagier R. J., DiBisceglie A. M., Shih J. W., Neuwald P. D., *J. Viral. Hepatitis*, **2**, 121—132 (1995).
7) Kern D., Collins M., Fultz T., Detmer J., Hamren S., Peterkin J. J., Sheridan P., Urdea M., White R., Yeghiazarian T., Todd J., *J. Clin. Microbiol.*, 34, 3196—3202 (1996).
8) Sanyl D., Kudesia G., Corbitt G., *J. Med. Microbiol.*, **35**, 291—293 (1991).
9) Kittigul L., Kahmoun P., Sujirarat D., Utrarachkij F., Chintpirom K., Chaichantanakit N., Vathanophas K., *Mem. Inst. Oswaldo Cruz.*, **96**, 815—821 (2001).
10) Li J. W., Wang X. W., Rui Q. Y., Song N., Zhang F. G., Ou Y. C., Chao F. H., *J. Virol. Methods*, **74**, 99—108 (1998).
11) Soares R. M., Durigon E. L., Bersano J. G., Richtzenhain L. J., *J. Virol. Methods*, **78**, 191—198 (1999).
12) Kilpatrick D. R., Nottay B., Yang C., Yang S., Silva E. D., Penaranda S., Pallansch M., Kew O., *J. Clin. Microbiol.*, **36**, 352—357 (1998).
13) Guideline I. H. T., "Proceeding of the Fourth International Conference on Harmonization (Brussels)," ed. by Arcy P. F. D., Harron D. W. G., The Queen's University of Belfast, W.&G. Baird Ltd., Northern Ireland, 1997, pp. 877—907.
14) Hudson J. B., Haugland R. P., Diwu Z., *Photochem. Photobiol.*, **65**, 352—354 (1997).
15) Ferrari M., Gualandi G. L., *Microbiologica*, **10**, 301—309 (1987).
16) Johnson K. L., Sarnow P., *J. Virol.*, **65**, 4341—4349 (1991).
17) LaBarre D. D., Lowry R. J., *J. Virol. Methods*, **96**, 106—126 (2001).
18) Watanabe W., Konno K., Ijichi K., Inoue T., Yokota T., Shigeta S., *J. Virol. Methods*, **48**, 257—265 (1994).
19) Mosmann T., *J. Immunol. Methods*, **65**, 55—63 (1983).
20) Chruscinski L., Dyba M., Jezowska-Bojczuk M., Kozlowski H., Kupryszewki G., Mackiewicz Z., Majewska A., *J. Inorg. Biochem.*, **63**, 49—55 (1996).
21) Sagripanti J., Routson L. B., Bonifacino A. C., Lytle D., *Antimicrob. Agents Chemother.*, **41**, 812—817 (1997).
22) Kümel G., Schrader S., Zentgraf H., Brendel M., *J. Gen. Virol.*, **71**, 2989—2997 (1990).
23) Arens M., Travis S., *J. Clin. Microbiol.*, **38**, 1758—1762 (2000).
24) Sujak I., Kawecki Z., *Acta Virol.*, **20**, 196—201 (1976).

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT•

Journal of Virological Methods 114 (2003) 11–19

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Virus concentration using polyethyleneimine-conjugated magnetic beads for improving the sensitivity of nucleic acid amplification tests

Koei Satoh[a], Akiko Iwata[a,b], Mitsuhiro Murata[c], Mikio Hikata[c], Takao Hayakawa[b], Teruhide Yamaguchi[b,*]

[a] *The Institute of Saitama Red Cross Center, Kamiochiai 8-3-41, Saitama 338-0001, Japan*
[b] *The National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan*
[c] *JSR Corporation, Tsukuba Research Laboratories, Miyukigaoka 25, Tsukuba, Ibaraki 305-0841, Japan*

Received 6 February 2003; received in revised form 18 August 2003; accepted 21 August 2003

Abstract

To enhance the sensitivity of virus detection by polymerase chain reaction (PCR) and reverse-transcriptional (RT)-PCR, we developed a novel virus concentration method using polyethyleneimine (PEI)-conjugated magnetic beads. PEI-magnetic beads adsorbed efficiently the enveloped viruses Sindbis virus and Herpes simplex 1 virus, and the nonenveloped virus SV-40, but not the nonenveloped viruses porcine parvovirus (PPV) or poliovirus, based on the PCR detection data. Furthermore, the infectivity in the supernatant of former viruses was reduced markedly after incubation with PEI-magnetic beads. Both real-time PCR and RT-PCR revealed that the DNA viruses were concentrated to a maximum of about 100 times the expected value, whereas the RNA viruses were concentrated over a thousand times, which was significantly more than expected. It was concluded that the PEI-magnetic beads are a superior novel means of concentrating viruses, with the exception of some non-enveloped viruses. The present method was found to enhance the sensitivity of virus detection by PCR and RT-PCR.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Magnetic beads; Nucleic acid amplification test; Polyethyleneimine

1. Introduction

Recently, several measures have been aimed at improving regulations regarding the viral safety of biological drugs, including plasma derivatives. The screening of starting materials, in-process materials, and final products for viruses is crucial for reducing the risk of viral contamination. In many countries, nucleic acid amplification tests (NATs) have been introduced recently into source material testing programs in order to screen for several viruses (Willkommen et al., 1999).

Polymerase chain reaction (PCR) is a highly sensitive method for the detection of virus genomes (Kwok et al., 1988; Larzul et al., 1988; Laure et al., 1988; Saiki et al., 1988). Recently, NAT methods other than PCR have been developed (Alter et al., 1995; Kamisango et al., 1999; Kern et al., 1996; Sarrazin et al., 2000; Wiedmann et al., 1994). These tests have been reported to be able to detect several copies of virus genomes. These screening tests are also thought to be useful for screening of cell therapy products, i.e., biotechnology products derived from cell lines and blood products. Furthermore, it has been hypothesized that if target viruses could be concentrated, the sensitivity of NATs, which are used to detect such viruses, would be enhanced.

The concentration of viruses has thus been attempted by many groups interested in enhancing the sensitivity of virus genome detection (Kanarek and Tribe, 1967; Li et al., 1998; Sanyal et al., 1991; Sanyl et al., 1991; Schloer and Breese, 1982; Stow and Wilkie, 1976). The ultra-centrifugation of viruses has been shown to be a superior method of concentration, but is very time-consuming and is not suitable for screening purposes. Polyethylene-glycol precipitation techniques are simple and render it easy to concentrate viruses, but the excess amount of extracted polyethylene-glycol hampers the PCR reaction. Genome-capture is superior for

Abbreviations: PEI, polyethyleneimine; HSV-1, Herpes simplex 1 virus; PPV, porcine parvovirus; VSV, vesicular stomatitis virus

* Corresponding author. Tel.: +81-3-3700-9373; fax: +81-3-3707-6950.
E-mail address: yamaguch@nihs.go.jp (T. Yamaguchi).

0166-0934/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2003.08.002

concentrating specific virus genomes, but immobilized probes for each virus must be prepared in order to capture many species of viruses. Therefore, the search for a more simple and feasible method of virus concentration has been ongoing.

In the present study, it was found that PEI-magnetic beads can trap many types of viruses, with the exception of some non-enveloped viruses. The usefulness of PEI-magnetic beads in enhancing the sensitivity of virus genome detection was thus the focus of the present study.

2. Materials and methods

2.1. Materials

Magnetic beads (IMMUTEX-MAG™; JSR Inc., Japan), were used throughout this study. IMMUTEX-MAG beads are acrylic latex beads with magnetite inside, and the surface of these beads is conjugated with carboxylic groups. The IMMUTEX-MAG beads used in the present study were 0.8 μm in diameter, and the surface charge density was 21 μmol/g. Polyethyleneimine (PEI, Mw 70,000) and 1-ethylene-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) were purchased from Wako Pure Chemical Inc., Japan. The primer sets used in the PCR and RT-PCR are shown in Table 1. The 5′-primer, 3′-primer, and fluorescence probes for the real-time PCR detection of HSV-1 are shown in Table 2.

2.2. Viruses

In the present study, the model viruses employed were those that have been used previously for viral clearance studies, as recommended by the international harmonized guideline (Guideline, 1997). Sindbis virus, SV-40 virus, Herpes simplex virus-1 (stain F) (HSV-1), and porcine parbovirus (strain 90HS; PPV) were kindly donated by Dr. Kohase (National Institute of Infectious Diseases). Vasicular stomatitis virus (strain NJ; VSV) was donated by Dr. Kita (Suntory Center Institute, Japan). Poliovirus (strain Sabin) was kindly donated by Dr. Koga (JCR Co., Japan). Vero (JCRB 0111) and CV-1 cells (JCRB 9049) were obtained from the Japanese Collection of the Research Bioresources (JCRB) Cell Bank. The ESK cells (embryonic swine kidney cell line) (Kawamura et al., 1988) were kindly donated by Dr. Koga. The supernatants of Vero cells infected with HSV-1, poliovirus (Sabin1), Sindbis virus, and VSV were used as the virus samples. CV-1 cells were infected with SV-40 virus, and then 5 days after infection, the supernatant was saved as the SV-40 sample. The supernatant of ESK cells infected with porcine parvovirus (PPV) was used as the PPV sample. In order to remove the cell debris from the collected virus suspension, each suspension was centrifuged at 3000 rpm for 10 min. After removing the cell debris, the resulting stock viruses were aliquoted and stored at −80 °C until use.

Table 1
Primer sets used for PCR and RT-PCR

	Primer set
HSV-1*1	5′-ATCCGAACGCAGCCCCGCTG-3′ 5′-TCTCCGTCCAGTCGTTTATCTTC-3′
SV-40*2	5′-ATAATTTTTTTGTATAGTAGTGCA-3′ 5′-GGAAAGTCCTTGGGGTCTTCTTACC-3′
Sindbis virus	5′-GGATTGGSTTYGAYACCACYCAGTTCATGT-3′ 5′-TGCCCYATGCGKAGYCCMGAAGAYCC-3′
VSV	5′-AGGATATGTCTACCAAGGCC-3′ 5′-GGTTATTTTGCAGAGGTGTCC-3′
PPV*3	5′-TTGGTAATGTTGGTTGCTACAATGC-3′ 5′-TATGTTCTGGTCTTTCCTCGCATC-3′
Poliovirus*4	5′-TGCGAGATACCACACAT-3′ 5′-ATCATGCTTTCAAGCATCTG-3′

Each primer set was prepared according to the method used in the original papers: HSV-1 (Kessler et al., 2000), SV-40 (Fedele et al., 1999), PPV (Soares et al., 1999) and poliovirus (Kilpatrick et al., 1998) (*1–*4).

Table 2
Primer and probe sets used for real-time PCR and real-time RT-PCR

	Primer and probe set
HSV-1	5′-GCGTCATGGTCATGGCAAG-3′ 5′-TTGACTCTACGGAGCTGGCC-3′ FAM-labeled 5′-TGGAGCTGATGCCGTAGTCGG-3′
Sindbis virus	5′-CAGGACGTCTATAACGCTCC-3′ 5′-GAGAACATGAACTGGGTGGTGTC-3′
VSV	5′-AGGATATGTFCTACCAAGGCC-3′ 5′-GGTTATTTTGCAGAGGTGTCC-3′
SV-40 virus	5′-ATAATTTTTTTGTATAGTAGTGCA-3′ 5′-GGAAAGTCCTTGGGGTCTTCTTACC-3′

DNA and RNA from Sindbis virus, VSV, and SV-40 virus were quantitatively determined using Syber Green PCR Master Mix™ (Applied Biosystems).

2.3. Coupling of polyethyleneimine with magnetic beads

PEI was immobilized covalently via its amino group to the magnetic beads by the EDC coupling method. One gram of magnetic beads was washed four times with 0.1 M HEPES buffer (pH 7.0) by a magnetic separation technique; the magnetic beads were trapped in a magnetic field (Magical Trapper™, Toyobo Co., Japan, Dynal MOC-1™, Dynal AS, Oslo), and after the aspiration of the solution, the beads were suspended in the same buffer. Finally, the washed beads were re-suspended in the same buffer at 50 mg/ml. The coupling reaction was initiated by the addition of 40.3 g EDC to the magnetic bead suspension, which was then vortexed thoroughly and incubated for 15 min under conditions of slow shaking. After the incubation, 200 mg of PEI dissolved in 10 ml of 0.1 M HEPES buffer (pH 7.0) was added to the reaction mixture. The reaction mixture was incubated subsequently under conditions of shaking for 2 h at room