資料２

別紙

HBV-DNA国内標準再評価のための共同研究
国内標準品候補品測定結果報告書

参加者施設　　施設名
担当者氏名
電話
メール

核酸抽出法

施設コード	
抽出試薬	キット: 自家法(原理):
抽出装置(使用した場合)	
試料、核酸溶液の量	希釈した試料(　　)μℓを使用し 核酸溶液(　　)μℓを調製する。 体外診断薬キットについては記入不要。試薬メーカーが代表して記入して下さい。

核酸増幅法

試薬	キット名: 又は 自家法(原理):
装置	
増幅する領域	体外診断薬キットについては記入不要。試薬メーカーが代表して記入して下さい。
1増幅反応当り使用する核酸の量	(　　)μℓ 体外診断薬キットについては記入不要。試薬メーカーが代表して記入して下さい。

HBV-DNA国内標準品再評価のための共同研究
国内標準品測定結果報告書

施設コード/参加施設名/担当者　施設コードXX/国立感染症研究所/村山　花子

測定結果(1回目)/測定日:

Sample	Dilution (neat, 10^{-1}, 10^{-2} etc)	*HBV Result (IU/mL)	Crossing point / Ct value (if applicable)	Comments
S	neat	記入上の注意参照		
	10^-1			
	10^-2			
C	neat			
	10^-1			
	10^-2			

*【記入上の注意】各測定系のキャリブレーター（プラスミド、合成核酸等）を用いて得た値を希釈した試料mL当りに換算して記入する。表示単位には各測定系で使用している単位（コピー、"IU"）を選択して記入する。

HBV-DNA国内標準品再評価のための共同研究

国内標準品測定結果報告書

施設コード/参加施設名/担当者

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Sample	Dilution (neat, 10^{-1}, 10^{-2} etc)	*HBV Result (IU/mL or copy/mL)	Crossing point / Ct value (if applicable)	Comments
S				
C				

*【記入上の注意】各測定系のキャリブレーター(プラスミド、合成核酸等)を用いて得た値を希釈した試料mL当りに換算して記入する。表示単位には各測定系で使用している単位(コピー、"IU")を選択して記入する。

HBV-DNA国内標準品再評価のための共同研究
国内標準品測定結果報告書

施設コード/参加施設名/担当者

測定結果(2回目)/測定日:

Sample	Dilution (neat, 10^{-1}, 10^{-2} etc)	*HBV Result (IU/mL or copy/mL)	Crossing point / Ct value (if applicable)	Comments
S				
C				

*【記入上の注意】各測定系のキャリブレーター(プラスミド、合成核酸等)を用いて得た値を希釈した試料mL当りに換算して記入する。表示単位には各測定系で使用している単位(コピー、"IU")を選択して記入する。

HBV-DNA国内標準品再評価のための共同研究

国内標準品測定結果報告書

施設コード/参加施設名/担当者

測定結果(3回目)/測定日:

Sample	Dilution (neat, 10^{-1}, 10^{-2} etc)	*HBV Result (IU/mL or copy/mL)	Crossing point / Ct value (if applicable)	Comments
S				
C				

*【記入上の注意】各測定系のキャリブレーター(プラスミド、合成核酸等)を用いて得た値を希釈した試料mL当りに換算して記入する。表示単位には各測定系で使用している単位(コピー、"IU")を選択して記入する。

平成26年度厚生労働科学研究委託事業（医薬品等規制調和・評価研究事業）
委託業務成果報告（業務項目）

NATの実施及び精度管理に関するウイルス学的研究

担当責任者　岡田義昭　埼玉医科大学病院　輸血・細胞移植部　部長

研究要旨

ヒトパルボウイルスB19（以下B19V）は、一過性の高いウイルス血症を呈する一方、供血者の40%～50%がB19Vに対する抗体を保有している。米国では原料血漿の基準として10^4IU/mLと過去の感染例を参考に決定されている。我々は、筋注用人免疫グロブリンを用いたin vitro感染系によって中和活性を評価し、10^4IU/mLが適した基準であることを実験的に示すことができた。

また、デングウイルスを検出するためにNAT検査が実施されているが、血液製剤の安全性確保の点から血清型別に実施するのではなくユニバーサルに検出できる系を考案する必要がある。そのために４つのデングウイルスの血清型に共通する塩基配列を検討した。各血清型を検出できたが、感度に100倍以上の差が認められ、更なる検討が必要であった。

A. 研究目的

輸血用血液を含めた血液製剤の安全性向上のためにHBV、HCV、HIVを対象とした核酸増幅検査が実施されているが、これらの検出感度は、検出試薬の感度を考慮して決定されていることが多い。一方、B19Vは一過性に高いウイルス血症を生じるが、感染者の多くが不顕性感染となるため献血者の40～50%がB19Vに対する抗体陽性と考えられている。そのため、血漿分画製剤の原料血漿におけるB19VのNAT検査は、高いウイルス血漿を排除するために実施されている。その一方で規格値を設定する際には、中和抗体の存在も考慮して決定する必要があると考えられる。米国のFDAでは、治験中に発生したS/D処理プラズマによるB19Vの感染事例の解析から感染が成立しなかった（抗体が陽転しなかった症例）ウイルス濃度を根拠に血漿分画製剤の原料血漿は10^4 IU/mL以下と規定している。欧州では、通常の血漿分画製剤用の血漿には規格は設定していないが、抗Dヒト免疫グロブリンは妊婦に投与することから原料血漿を10^4 IU/mL以下と規定している。我々は、B19Vの国内標準品が設定されたことから血漿分画製剤の原料血漿に規格を設定する場

合、どれくらいのウイルス量が適切であるのかB19Vの感染系を用いて評価を試みた。また、今年度デングウイルスが国内で発生したことを受け、デングウイルスを検出するNAT検査法を検討し、４つの血清型をユニバーサルに検出できるNATを試みた。

B.研究方法

1) B19V感染性の評価

B19Vは細胞に侵入するとDNAからRNAに転写され、数カ所でスプライシングされて最終的にタンパク質に翻訳される。この性質を利用して B19V を感染させた細胞からスプライシングされたRNAが検出できた場合に感染性を有すると判断した。塩基配列の584番と585番の間で切れ、同様に2087番と2088番の間で切れて584番と2088番が結合したRNAができるためスプライシング部位を挟むようにRTと1stPCR、及び２ndPCR 用の２組のプライマーを用いた。増幅産物はスプライシングされてできる RNA 由来の cDNA と添加したB19V由来のDNAとを明確に区別できる。

2) 筋注用人免疫グロブリン製剤によるB19V中和活性の評価

血漿分画製剤の製造用血漿が入手できないので代用として市販されている筋注用人免疫グロブリン製剤を用いた。筋注用人免疫グロブリン製剤は IgG を 150mg/mL 含有し、血漿の約 10 倍の濃度である。5%アルブミン 400μL に筋注用人免疫グロブリン製剤 50μL、5%アルブミンで 10^{-1}～10^{-8} に稀釈したB19V 50μLを加え計500μL調整し、4℃で混和させながら２時間反応させた。中和させた溶液から別なチューブに200μL取り、そこへ $3X10^5/50\mu L$ に調整した F10（KU812 由来、当研究室で分離・維持している）添加し、ローテーターで回転させながら室温で1時間ウイルスを感染させた。感染後、1mLの10%FCS-RPMI（エリスロポイチン3単位/mL含む）を加え2日間培養し、遠心にて細胞を回収した（図１）。細胞にRNAsol　を添加してRNAを抽出し、最終的にRNAは12μLの蒸留水に溶解した。RT及び 1_{st}PCRはPrimScript One Step RT-PCR Kit Ver.2(タカラ)を用い、RNA10μを用いた。2_{nd}PCRは5μLの1stPCR産物を用いた。抗 B19V 陰性の免疫グロブリン製剤はないので中和活性なしのコントロールとして5%アルブミンを用いた。スプライシングされたRNAが増幅された最大稀釈率を求めた。

なお、筋注用人免疫グロブリン製剤は３つの異なる製造所が製造した製剤を用いた。

3)NAT によるデングウイルス検出のためのユニバーサルプライマーの検討

デングウイルスは４つの血清型があり、それぞれ特異的なプライマーを用いて検出が実施されている。従って４つの NAT 検査を実施していることになる。血液製剤の安全性確保のためには、デングウイルスの有無を効率良く評価できることが優先することからユニバーサルに血清型を検出できるプライマーを検討した。文献からこれまで報告されたプライマーとデングウイルスの遺伝子配列からプライマーを決定し、各血

清型由来のデングウイルス RNA の検出を試みた。

C. 研究結果

1) 筋注用人免疫グロブリン製剤による B19V 中和活性の評価

人 IgG を含まない 5%アルブミン（中和活性がない場合のコントロール）では、10^7 倍まで B19V の感染性が認められた。一方、筋注用人免疫グロブリン製剤では、検体間に差がなく 10 倍稀釈あるいは 100 倍稀釈まで感染性が認められた。同一検体を反復測定しても 10 倍稀釈あるいは 100 倍稀釈まで感染性が認められた。 100 倍稀釈では感染性を示すシグナルは 10 倍稀釈に比べて弱い傾向があった。以上から 15mg の人 IgG によって感染価は少なくても 5Log 中和されることが示された（図２）。

2) NAT によるデングウイルス検出のためのユニバーサルプライマーの検討

Am. J. Trop. Med. Hyg. vol. 56. 424-429. 1997 及び J. Clin. Micro 2323-2330. 2002 を参考にして RT と 1st PCR は、10,406～10,432 と 10,674～10,694、２ nd PCR は 10,406～10,423 と 10,617～10,634 のプライマーを用いて semi-nested PCR を行なった。４つの血清型のどの型にも合うように血清型間で異なる塩基配列の部分は ミックス配列とした。ほぼ同量のデングウイルス RNA が存在すると考えられる各血清型のデングウイルスの検出を行なったところ、各血清型デングウイルスは検出できたが、血清型３と４に比較して血清型１は 1/10、血清型２は 1/100 以下の感度であった。

D. 考察

B19V は多くの場合不顕性感染となるため抗体保有率は、大人の 40～50%と言われている。抗 B19 抗体は中和活性があることが知られている。米国の S/D 処理した新鮮凍結血漿による B19V 感染事例から、10^7 IU/mL 以上では感染が生じ、10^4IU/mL 以下では感染例がなかったこと知られている。これを基に米国では 10^4IU/mL 以下を原料血漿の規格値としている。一方、症例数は少ないが輸血による B19V 感染の解析から 10^3IU/mL 以下では感染した症例がないことも報告されている。全血から感染したとすると１バッグの血漿量から約 2X10^5IU が、B19V 抗体陰性の人が感染する最少の量と推定できる。原料血漿の基準を 10^4 IU/mL とし、原料血漿プールの容量を 3000L とした場合、プール全体で最大 3X10^{10}IU の B19V が混入していることになるが、我々の実験結果から血漿中に存在する抗体によって 5Log 感染性がなくなると推定できる。その結果、プール血漿中の感染性ウイルスは 3X10^5IU となり最大１人を感染させる量が残存するだけになる。製造工程によってさらに B19V は除去・不活化されるので最終製剤にまで感染性ウイルスが残存する可能性は極めて低くなると考えられる。

我々は、これまで B19V の感染性の評価を行い、方法を改良してきた。今回、製造工程を考慮し、中和反応を 4℃で行なった。また、ヒト免疫グロブリンと B19V を反応させ

る容量を 500μL に増量することで混和がし易いようにした。また、感染させる F10 細胞を 1.5 倍にしたことで RNA の沈殿が明瞭になり操作が容易になった。その結果、安定した結果が得られるようになった。

デングウイルス検出のためのユニバーサルプライマーの検討では、今年度のプライマーでは血清型間で2Log以上の感度の差が生じた。特に２型の感度が悪かったので塩基配列を再検討し、感度の向上をはかる必要がある。

E. 結論

B19V の中和活性測定法を改良し、血漿相当量の人免疫グロブリンによって約5Log中和されることを明らかにし、原料血漿の基準 10^4IU/mL は、実験的にも適切であることが示された。また、デングウイルスの血清型全てを検出するためのユニバーサルプライマーを検討したが、血清型によっては感度が 1/100 以下となり、更なる改良が必要である。

F.　健康危機情報

なし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

なし

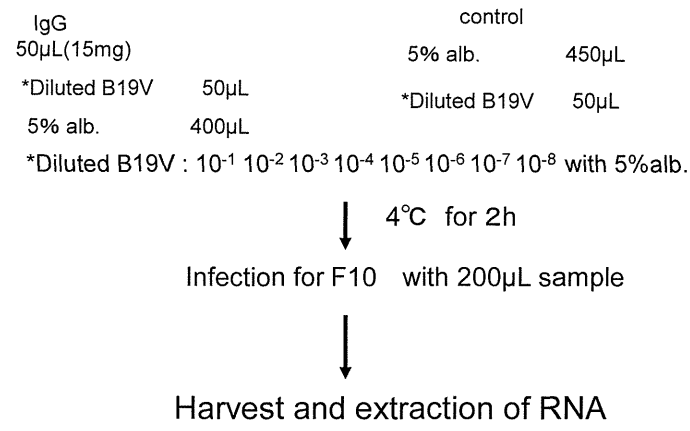

図1.人免疫グログリン製剤による B19Vの中和活性

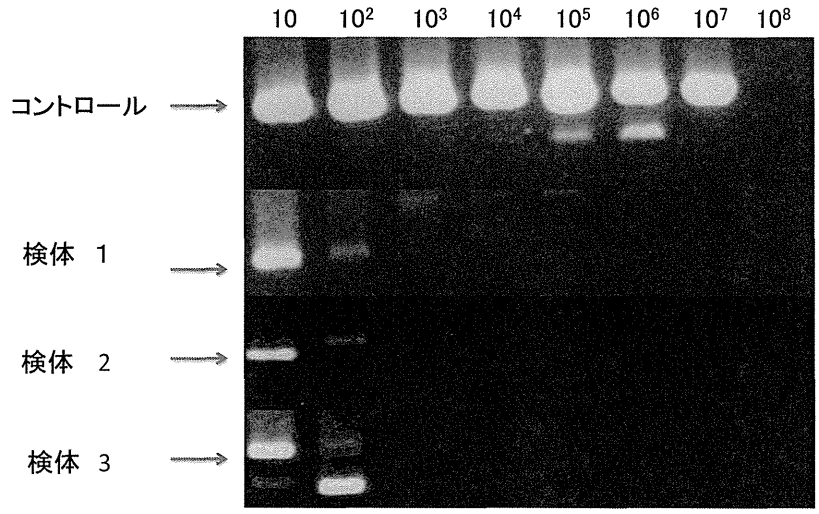

図2.人免疫グロブリンによるB19V中和活性

学会等発表実績

1. 学会等における口頭・ポスター発表

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	美子、山田攻、小林清子、池淵研二、岡田義昭			
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Identification and characterization of cancer stem cells in an HBZ transgenic mouse model of ATL	Kuribayashi W, Mizukami T, Takizawa K, Kuramitsu M, Asada Y, Iwama A, Matsuoka M, Hamaguchi I	第76回日本血液学会学術集会	2014.10	国内

2. 学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
A shared regulatory perspective on deferral from blood donation of men who have sex with men (MSM).	Epstein J, Ganz PR, Seitz R, Jutzi M, Schaerer C, Michaud G, Agbanyo F, Smith G, Prosser I, Heiden M, Saint-Marie I, Oualikene-Gonin W, Hamaguchi I, Yasuda N	Vox Sang	2014年	国外
Development of an Infectious Surrogate Hepatitis C Virus Based on a Recombinant Vesicular Stomatitis Virus Expressing Hepatitis C Virus Envelope Glycoproteins and Green Fluorescent Protein.	Okuma K, Fukagawa K, Tateyama S, Kohma T, Mochida K, Hiyoshi M, Takahama Y, Hamaguchi Y, Hirose K, Buonocore L, Rose JK, Mizuochi T, Hamaguchi	Jpn J Infect Dis.	2015年	国内
Identification of TL-Om1, an Adult T-Cell Leukemia (ATL) Cell Line, as Reference Material for Quantitative PCR for Human T-Lymphotropic Virus 1.	Kuramitsu M, Okuma K, Yamagishi M, Yamochi T, Firouzi S, Momose H, Mizukami T, Takizawa K, Araki K, Sugamura K, Yamaguchi K, Watanabe T, Hamaguchi I,	J. Clin. Microbiol.	2015年	国外
輸血用血液における病原体不活化技術の現状と新規技術の開発	岡田　義昭	検査と技術	2014年	国内
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REPORT

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A shared regulatory perspective on deferral from blood donation of men who have sex with men (MSM)†

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National Regulatory Authorities (NRAs) establish deferral criteria for donors with risk factors for transfusion transmissible infections (TTI). In most jurisdictions, epidemiological data show that men who have sex with men (MSM) have a significantly higher rate of TTI than the general population. Nevertheless, changes from an indefinite donor deferral for MSM have been considered in many countries in response to concerns over a perceived discrimination and questioning of the scientific need. Changes to MSM donor deferral criteria should be based on sound scientific evidence. Safety of transfusion recipients should be the first priority, and stakeholder input should be sought.

Key words: blood collection, donors, epidemiology, motivation/recruitment, transfusion medicine (in general).

Background

To protect patients receiving blood components against exposure to transfusion transmissible infectious agents (TTI), National Regulatory Authorities (NRAs) require blood collection establishments to screen blood donors through a combination of a health questionnaire and laboratory testing procedures. Available laboratory tests are highly sensitive for detection of the major TTI. However, testing is not available for all significant TTI; laboratory tests cannot reliably detect donor infections with TTI during the early infection period; and procedural errors are possible [1–4]. Therefore, deferral from donation of persons with increased risk of contracting TTI takes on importance as a key safety measure to protect transfusion recipients against TTI.

To be effective as a safety measure, the health questionnaire helps to identify risk factors, including certain sexual behaviours, whose association with TTI is established through epidemiological studies. In particular, male sex with other males has been associated with increased risk of TTI [5–9]. However, policies to defer blood donors indefinitely based on a history of male sex with another male have been controversial in many countries due to perceptions of discrimination. Questions also have been raised whether such a deferral is necessary or effective [10–17]. In principle, longer durations of deferral for MSM may not inherently result in lower risks of TTI in

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donors unless those criteria select for donors at lower risk of recent infection. Lower TTI risk was correlated with an MSM abstinence period of longer than 5 years in one study [18].

National Regulatory Authorities that establish risk-based donor deferral criteria have emphasized that these policies attempt to exclude potential donors with identified risk factors. In the case of MSM, the donor deferral is based on the risk that is associated with the behaviour (i.e. sexual behaviours that constitute an elevated risk to acquire TTI) and is not based on the sexual orientation per se of the donor. The consequent application of this criterion is of high importance for the safety of the blood supply and is not meant to discriminate against any individual or group. In most jurisdictions, epidemiological and public health data have shown and continue to show that men who have had sex with other men have a significantly higher rate than the general population of acquiring, carrying and transmitting certain infectious agents, such as HIV, hepatitis and potentially other TTI [5–9].

Because of the general difficulty in assessing individual risk related to sexual behaviour [19], many NRAs have chosen to apply a uniform MSM deferral. Similar considerations regarding assessing individual risk have been applied to exclude donors with a history of illicit injection drug use, sex workers and donors with exposure in certain geographical areas where risk of acquiring TTI is increased.

Current situation

Similar epidemiological data showing increased TTI risk among MSM exist in various jurisdictions, and many NRAs worldwide have a policy of indefinite deferral for MSM. Although there are variable results, many modelling studies have shown that in changing the deferral period from indefinite to 1, 5 or 10 years, a very small absolute increase in risk cannot be ruled out [17, 18, 20–22]. In a recent retrospective analysis in Australia, the HIV prevalence of donors was found not to have increased following a change to a time-based 12-month deferral [23]. A number of countries have changed the MSM deferral from an indefinite to a time-based deferral. Among these countries are Australia, Canada and Japan who have contributed to this publication and share the regulatory perspective described herein. Some other countries have replaced a uniform deferral for MSM behaviour with donor questioning to identify recent high-risk sexual exposure (e.g. Italy, Mexico, Poland, Russia, Spain).

The available scientific data and the policies regarding deferral on the basis of sexual behaviour in different jurisdictions have recently been analysed [24, 25]. In some countries, studies have been undertaken to better understand the attitudes and behaviour of MSM towards donation, the adequacy of the donor history questionnaire to identify donors at increased risk of TTI, and the impact of potential or actual changes from a policy of indefinite deferral for MSM. Similar to the conclusion of another report [9], the authors noted that overall risk to transfusion safety would be influenced to a large extent by the degree of adherence by MSM to revised deferral criteria [23, 26].

A less restrictive policy on blood donation by MSM has been projected to produce only a small public health benefit through an increase in blood donations [16]. Any projected increase to the blood supply has not been thought by many NRAs to be sufficient to warrant a policy change at this time. Many NRAs have taken the view that any preventable projected increase in risk to the blood supply from MSM donation should not be imposed on recipients of blood components in the absence of a countervailing benefit to recipients.

Considerations for decision-makers

Scientific evidence

Decision-making for NRAs is predicated on relative benefit to risk considerations informed by current scientific understanding and available evidence. Factors that may be relevant when considering changes from an indefinite MSM deferral could be as follows:

(1) Evidence from other jurisdictions which have changed the deferral from indefinite to a time-based deferral indicating that there has been no increase in risk (e.g. based on TTI marker rates, especially window period infections, prevalence in first time donors, and rates of seroconversion in repeat donors) and no observed increase in the frequency of pathogen transmissions based on sound haemovigilance monitoring.
(2) Evidence that the current deferral policy is not effective at reducing risk of TTI. In this context, the effectiveness of health questionnaires should be assessed and options for improvements should be explored.
(3) Evidence from well-designed studies that revised donor selection criteria allowing blood donations by some men with a prior history of sex with another man would not be likely to increase the risk of TTI.
(4) Consideration of risk mitigating steps that could be implemented in conjunction with a change to MSM deferral criteria so as to improve current blood safety controls (e.g. predonation testing, quarantine hold pending postdonation testing for some products, single unit nucleic acid testing and pathogen reduction).

(5) Evidence that changes in deferral criteria will not increase risk from inadvertent release of infectious units from quarantine (quarantine release errors).

Societal considerations

National Regulatory Authorities function within a broader framework of society wherein social attitudes, perceptions and priorities are relevant to and may influence policy decision-making. NRAs and other relevant government bodies should seek to receive comments from all stakeholders, when contemplating a change to the MSM deferral policy. The overriding consideration should be that a change in policy should not increase risk to transfusion recipients. However, de minimus changes in risk may be acceptable in the context of larger societal benefits. Social factors affecting the overall safety and adequacy of the blood supply such as compliance with deferral criteria and public willingness to donate blood may be part of the assessment of risks and benefits of a policy change. The discussion should include whether the public is prepared to accept some added risk of transfusion for a possible benefit of reducing a perceived discrimination against MSM.

Conclusion and recommendations

Based on the principle that the safety of blood transfusion for recipients should be the primary concern of the NRA, the authors recommend careful deliberation in considering a less restrictive blood donor deferral for MSM.

Donor deferrals based on sexual behaviour play an important role in preventing disease transmission from TTI. However, despite the clear association of TTI risk with a history of male sex with other males, policies to indefinitely defer MSM from blood donation as a blood safety measure are controversial in many jurisdictions both on scientific and societal grounds. Consequently, many NRAs are now reviewing their current policy of MSM deferral, some have implemented, and others are considering a modification of their existing policy. Time-limited deferrals have been implemented by some NRAs, at least partly in consideration of country-specific socio-political concerns including alleviation of a perceived discrimination and possibly based on tolerance of a potential small increase of risk.

Modelling studies suggest that changes from indefinite to time-limited deferrals will increase TTI risk unless accompanied by an increase in adherence to the deferral criteria by MSM. In particular, the motivations of MSM to donate and the likelihood that a modification of the donor criteria could change the degree of MSM compliance need to be assessed. Implementation of revised deferral criteria for MSM in Australia did not result in an increase in the rate of HIV-positive donations [23]. A more recent study in the same country was interpreted to confirm the previous finding of a high degree of compliance by MSM with a 12-month deferral period [26]. Whether the same outcome could be achieved in different settings cannot be predicted due to differences in epidemiology of infections and societal norms. Improvements in the effectiveness of health questionnaires may contribute to blood safety in conjunction with a policy change on deferral for history of MSM.

The authors believe that changes to the donor deferral for history of MSM should be evidence driven and based on sound science. Consequently, the authors recommend that NRAs consider the following points as part of the overall decision process:

(1) Safety of transfusion recipients should be the first priority of the NRA.
(2) Appropriate studies should be conducted to assess the potential impacts of policy changes, to validate candidate risk mitigation strategies implemented in association with policy changes and to monitor the safety outcomes of a policy change.
(3) Stakeholder input should be sought through a formal process designed to enable all viewpoints to be considered constructively by the NRA.
(4) In order to inform future decision-making, also relating to MSM deferral policy, individual jurisdictions should continuously assess haemovigilance data regarding the risk of TTI in donors. Emphasis should be given to monitoring adherence to donor selection criteria, especially following changes in donor questionnaires or deferral periods.

In conclusion, the authors believe that NRAs should follow the same principles in addressing the issue of donor deferral for MSM, namely priority for patient safety, evaluation of scientific data, consideration of the local epidemiology and societal circumstances, as well as stakeholder input, which may be quite different between countries. However, the authors recognize that based on such differences in the various jurisdictions, decisions based on the criteria stated above may result in different outcomes [22, 26, 27].

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Identification of TL-Om1, an Adult T-Cell Leukemia (ATL) Cell Line, as Reference Material for Quantitative PCR for Human T-Lymphotropic Virus 1

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Quantitative PCR (qPCR) for human T-lymphotropic virus 1 (HTLV-1) is useful for measuring the amount of integrated HTLV-1 proviral DNA in peripheral blood mononuclear cells. Many laboratories in Japan have developed different HTLV-1 qPCR methods. However, when six independent laboratories analyzed the proviral load of the same samples, there was a 5-fold difference in their results. To standardize HTLV-1 qPCR, preparation of a well-defined reference material is needed. We analyzed the integrated HTLV-1 genome and the internal control (IC) genes of TL-Om1, a cell line derived from adult T-cell leukemia, to confirm its suitability as a reference material for HTLV-1 qPCR. Fluorescent *in situ* hybridization (FISH) showed that HTLV-1 provirus was monoclonally integrated in chromosome 1 at the site of 1p13 in the TL-Om1 genome. HTLV-1 proviral genome was not transferred from TL-Om1 to an uninfected T-cell line, suggesting that the HTLV-1 proviral copy number in TL-Om1 cells is stable. To determine the copy number of HTLV-1 provirus and IC genes in TL-Om1 cells, we used FISH, digital PCR, and qPCR. HTLV-1 copy numbers obtained by these three methods were similar, suggesting that their results were accurate. Also, the ratio of the copy number of HTLV-1 provirus to one of the IC genes, RNase P, was consistent for all three methods. These findings indicate that TL-Om1 cells are an appropriate reference material for HTLV-1 qPCR.

Human T-lymphotropic virus 1 (HTLV-1) was the first retrovirus to be found in humans (1, 2). HTLV-1 is a cause of adult T-cell leukemia (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1-associated uveitis (3). Areas where HTLV-1 is endemic are distributed across several different regions, including southern Japan, the Caribbean, South America, and tropical Africa (4, 5). A recent report has shown that the area affected by this infection has expanded from the southern part of Japan to the entire country, particularly the Tokyo metropolitan area (6). Diagnostic tests for HTLV-1 infection are performed mainly with serological assays, such as enzyme-linked immunoabsorbent assay, particle agglutination assay, and Western blotting. Recently, another diagnostic test has been developed. Quantitation of integrated proviral DNA in peripheral blood (proviral load [PVL]) can be performed by quantitative PCR (qPCR) as a risk assessment for ATL or HAM/TSP (7, 8).

A few studies reported that several samples were positive for viral DNA when tested by PCR even though those samples had been found seroindeterminate for HTLV-1 when tested by Western blotting (9, 10). Their results suggest that HTLV-1 qPCR could be used as an additional test to confirm infection in seroindeterminate samples.

Although many laboratories have developed qPCR methods for HTLV-1 detection in Japan, a wide variety of testing methods are used. For example, the target region, primers and probes, and internal control (IC) genes vary among the laboratories (8, 11–15). These variations lead to significant differences in HTLV-1 PVL when these laboratories measure the same samples (16). As a consequence of these differences, comparison of quantitative data between laboratories will continue to be difficult without standardization.

One possible solution is to establish a reference material, which is indispensable for standardizing multicenter test results. The target material for HTLV-1 qPCR is genomic DNA (gDNA) from peripheral blood mononuclear cells (PBMCs). Therefore, HTLV-1-infected cells would be an ideal source for a reference material. To date, many cell lines from ATL patients have been established, but few of them have been well characterized for the genomic features associated with reference materials for HTLV-1 qPCR.

In this study, we investigated the genomic structure of one of these ATL cell lines, TL-Om1, to establish it as a reference material for HTLV-1 nucleic acid amplification techniques (NATs), namely, HTLV-1 clonality, karyotyping, proviral sequencing, integration sites, and determination of gene copy number of HTLV-1 and cellular genes for IC.

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TABLE 1 Primers used for qPCR of HTLV-1 and IC genes

Target gene	Forward name	Forward sequence	Reverse name	Reverse sequence	Size (bp)	Primer correction factor	
						Plasmid	gDNA
HTLV-1 gene	LTR202F	ACAATGACCATGAGCCCCAAA	LTR202R	TTAGTCTGGGCCCTGACCT	101	0.9869	
	LTR215F	GCTCGCATCTCTCCTTCAC	LTR215R	AGTTCAGGAGGCACCACA	102	0.9942	
	LTR005F	CCTGACCCTGCTTGCTCAAC	LTR005R	TCAGTCGTGAATGAAAGGGAAAG	99	0.9917	
	056F	TAGTCCCACCCTGTTCGAAATG	056R	GCCAGGAGAATGTCATCCATGT	105	1.0013	
	084F	CCTGCCCCGCTTACTATCG	084R	GGCATCTGTGAGAGCGTTGA	102	0.9922	
	153F	TTGTCGCGCTACTCCTTCTTG	153R	AGGGATGACTCAGGGTTTATAAGAGA	118	0.9792	
	pX2-S[a]	CGGATACCCAGTCTACGTGTT	pX2-AS[a]	CAGTAGGGCGTGACGATGTA	100	0.9944	
RNaseP (RPPH1) gene	RPPH1-05F	TATGCACAATTATGTAATCCCCAAA	RPPH1-05R	CCAGCTCCCTATAACCTGCACTT	100	1.0025	1.0012
	RPPH1-08F	GCCGGAGCTTGGAACAGA	RPPH1-08R	AATGGGCGGAGGAGAGTAGTCT	109	0.9956	0.9937
	RPPH1-12F	AGGAAGCCCACGAAAATTCTAATT	RPPH1-12R	GTCCCCATACTCGGTGATTCTC	101	1.0019	1.0052
Albumin (ALB) gene	ALB-07F	TGCAATGAACACAGGAGAGCTACTA	ALB-07R	CCACCCAGGTAACAAAATTAGCAT	103	0.9971	0.9964
	ALB-19F	CCTGATGCTTCTCAGCCTGTT	ALB-19R	TCCATTTAAGAGTGTGTGTGGTAGGT	100	1.0019	1.0045
	ALB-26F	TGCATTGCCGAAGTGGAAA	ALB-26R	CCTCAGCATAGTTTTTGCAAACA	100	1.0038	1.0078
β-Actin (ACTB) gene	ACTB-06F	TCTGGTGTTTGTCTCTCTGACTAGGT	ACTB-06R	CCGCTTTACACCAGCCTCAT	100		0.9965
	ACTB-12F	TCCTGGGTGAGTGGAGACTGT	ACTB-12R	CCATGCCTGAGAGGGAAATG	107		1.0016
	ACTB-21F	AGCATCCCCCAAAGTTCACA	ACTB-21R	GGACTTCCTGTAACAACGCATCT	101		1.0106
CD81 gene	CD81-01F	GACACATCCCAAGGGTGCTT	CD81-01R	GGACTCAGTTCTCAATGCTTTGC	107		1.0015
	CD81-10F	ACCACGCCTTGCCCTTCT	CD81-10R	GAATCACGCCACTTCCATAACTG	111		1.0021
	CD81-21F	GGTGCACACAGCATGCATTT	CD81-21R	GTGCGCCTCTGGGTAATCAT	102		1.0009
β-Globin (HBB) gene	HBB-11F	TTGGACCCAGAGGTTCTTTGAG	HBB-11R	GGCACCGAGCACTTTCTTG	103		1.0021
	HBB-15F	AGCAGCTACAATCCAGCTACCAT	HBB-15R	GAGGTATGAACATGATTAGCAAAAGG	105		1.0033
	HBB-24F	CCCACCCCAAATGGAAGTC	HBB-24R	AGCACCATAAGGGACATGATAAGG	104		1.0111
RAG-1 gene	RAG1-03F	GCAATCCCATTTGTCCACTTTT	RAG1-03R	TCCCACTGGCCTGCATTACTA	100		1.0045
	RAG1-27F	GAAGTTTAGCAGTGCCCCATGT	RAG1-27R	ACGGGCAGTGTTGCAGATG	100		1.0006
	RAG1-32F	TCAAAGTCATGGGCAGCTATTGT	RAG1-32R	AGGGAATTCAAGACGCTCAGAA	100		0.9993

[a] Primer sequences were previously reported in reference 11.

MATERIALS AND METHODS

Cells and gDNA preparation. Jurkat clone E6-1 cells were obtained from the American Type Culture Collection. HUT102 and SLB-1 cells, which are HTLV-1-infected cell lines, were a kind gift from Masahiro Fujii (Division of Virology, Niigata University Graduate School of Medical and Dental Sciences). PBMCs were kindly provided by the Japanese Red Cross or purchased from AllCells (Alameda, CA, USA). TL-Om1 cells, an ATL-derived cell line established by Sugamura et al. (17), were maintained in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 2 mmol/liter L-glutamine, and 10 ng/ml interleukin-2 (PeproTech, London, United Kingdom). Jurkat, HUT102, and SLB-1 cells were maintained in RPMI 1640 containing 10% FBS supplemented with 100 U/ml penicillin-streptomycin and 2 mmol/liter L-glutamine. DNA was extracted using a QIAamp DNA blood mini or maxi kit (Qiagen, Valencia, CA, USA).

Southern blotting. Southern blotting was performed by SRL Inc. (Tokyo, Japan). DNA was digested with EcoRI and PstI and separated on a 0.8% agarose gel as previously reported (18, 19). DNA was transferred onto nylon membranes (Roche, Mannheim, Germany). The membrane was hybridized with digoxigenin (DIG)-labeled HTLV-1 probe at 42°C overnight. DNA fragments for HTLV-1 probes were obtained from Oncor Inc. (Gaithersburg, MD, USA). Sense and antisense HTLV-1 DNA probes were prepared by random primed labeling using a DIG-High Prime kit (Roche). After the membrane was washed, HTLV-1 probe signals were obtained using a DIG luminescent detection kit (Roche).

FISH analysis. To stop the cell cycle at M phase, Colcemid (Sigma) was added to the cell culture medium at a concentration of 0.02 μg/ml and incubated for 1 h. Cells were harvested and washed with phosphate-buffered saline (PBS). After treatment with 0.075 M KCl hypotonic solution at 37°C for 1 h, cells were fixed with a solution containing acetic acid and methanol (3:1). Cells were fixed to a glass slide and dried. The complete HTLV-1 genome inserted in pUC18 (15) was used as a probe for provirus, bacterial artificial chromosome (BAC) clone RP11-919G18 was used as a probe for the albumin (ALB) gene, and BAC clones CTD-2326H15 and RP11-203M5 were used as probes for the RNase P (RPPH1) gene. BAC clones were selected from NCBI (http://www.ncbi.nlm.nih.gov/clone/) and were purchased from Advanced Geno Techs Co. (Tsukuba, Japan). The probe for 1q44 was commercially prepared by Chromosome Science Labo Inc. (Sapporo, Japan). For the detection of ALB and RPPH1 genes, the BAC clones were labeled with cyanine 3 (Cy3) and Cy5, respectively. For the detection of provirus, the DIG-labeled probe was prepared by the nick translation method. The probe was hybridized to the sample at 70°C for 5 min, followed by incubation at 37°C overnight. The probe was stained with anti-DIG-Cy3 antibody. Signals were detected by a Leica DMRA2 system and analyzed with Leica CW4000 fluorescent *in situ* hybridization (FISH) software (Wetzlar, Germany).

Splinkerette PCR analysis. Splinkerette PCR was performed as previously reported (20). The first-round PCR was performed as indicated in reference 20. The second-round, nested PCR was performed using the HTLV-1 long-terminal-repeat (LTR)-specific primer. The nested PCR product was loaded onto 3% Tris-acetate-EDTA buffer (TAE) agarose gels. Two distinct DNA bands were cut from the agarose gel and purified using a QIAquick gel extraction kit (Qiagen). After thymine and adenine (TA) cloning, each band was sequenced by the Sanger method (21).

Inverse PCR analysis. TL-Om1 gDNA was digested with BamHI or XbaI. Digested DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Briefly, 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol were added to the sample. After centrifugation at $2 \times 10^4 \times g$ for 15 min, the DNA pellet was washed with 70% ethanol and then air dried. Purified DNA was self-ligated using a Ligation-Convenience kit (Nippon Gene, Tokyo, Japan). Ligated DNA was purified again by phenol-chloroform extraction followed by ethanol precipitation. PCR was performed with KOD FX (Toyobo, Osaka, Japan). The PCR mixture contained 20 ng gDNA, 0.4 mM forward and reverse primers, 1 mM deoxynucleoside triphosphate (dNTP), 1× KOD FX buffer, and 0.5 U KOD FX in a total volume of 25 μl, in duplicate. The forward primer sequence was 5′-ACAAATACACCTTGCAATCCTATG G-3′, and the reverse primer sequence was 5′-CGCTTGGGAGACTTCT TGCT-3′. PCR mixtures were denatured at 94°C for 2 min, followed by 34 cycles of 98°C for 10 s and 68°C for 10 min. PCR products were loaded onto 0.8% agarose gels and detected by LAS-3000 (Fujifilm, Tokyo, Japan).

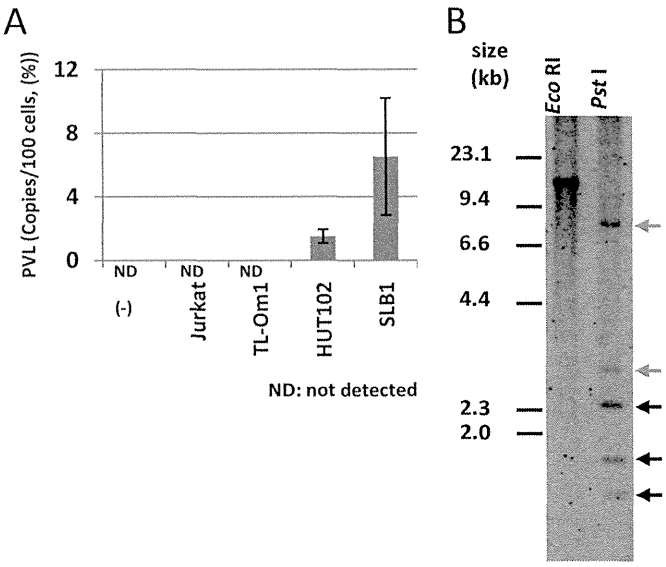

FIG 1 Infectivity and clonality of HTLV-1 provirus in TL-Om1 cells. (A) Mitomycin C-treated Jurkat, TL-Om1, HUT102, and SLB1 cells were cocultured with Jurkat cells. PVL (%) was measured 2 weeks later by qPCR. (B) gDNA from TL-Om1 cells digested with EcoRI or PstI was subjected to Southern blotting probed by the full HTLV-1 genome. Three black arrows show bands for typical HTLV-1 genomic sequences; two gray arrows show bands for host genomic sequences ligated to the HTLV-1 genome. Because the EcoRI site is not included in the HTLV-1 sequence, the number of bands indicates the number of clones in the cells. Detection of two gray bands indicates that there is a pair of 5′ and 3′ HTLV-1 genomes conjugated with the host genome, signifying that the HTLV-1 provirus is monoclonal. On the other hand, detection of more than two gray bands indicates that it is multiclonal.

Genomic long PCR. Genomic long PCRs were performed using KOD FX (Toyobo). Primers are listed in Table S1 in the supplemental material. The conditions for the PCR mixture and thermal cycling program were the same as those for the inverse PCR analysis.

DNA sequencing analysis. The genomic long PCR and inverse PCR products were purified by a GenElute PCR Clean Up kit (Sigma). Direct sequencing was performed using a BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence primers are listed in Table S2 in the supplemental material. Sequences were read and analyzed using a 3120× genetic analyzer (Applied Biosystems).

Synchronized qPCR analysis. The primers used for the synchronized qPCR amplification are listed in Table 1. The PCR mixture was prepared with SYBR premix *Ex Taq* II (TaKaRa, Tokyo, Japan) containing 100 ng gDNA and 0.4 mM forward and reverse primers in a total volume of 15 μl, in triplicate. PCR was performed according to the manufacturer's protocol. The ΔC_T(RPPH1) value (where C_T is threshold cycle) was calculated by the following equation: ΔC_T(RPPH1) = average C_T of target gene primer results − average C_T of RPPH1. The gene copy number was calculated by the following equation: target gene copy number (N) = copy number determined by FISH $\times 2^{-\Delta CT(\mathrm{RPPH1})}$. Using normal PBMCs or plasmids, the primer correction factor, which can compensate for small differences in amplification efficiency among different primers, was calculated. The correction factor was determined by the difference of each C_T