薬事・食品衛生審議会
平成２３年度　第４回　血液事業部会運営委員会

議 事 次 第

日時：平成２４年３月１４日（水）
１４：００～１６：００
場所：厚生労働省１２階 専用第１５・１６会議室

議題：

1. 議事要旨の確認
2. 感染症定期報告について
3. 血液製剤に関する報告事項について
4. 日本赤十字社からの報告事項について
5. その他

配付資料：

座席表

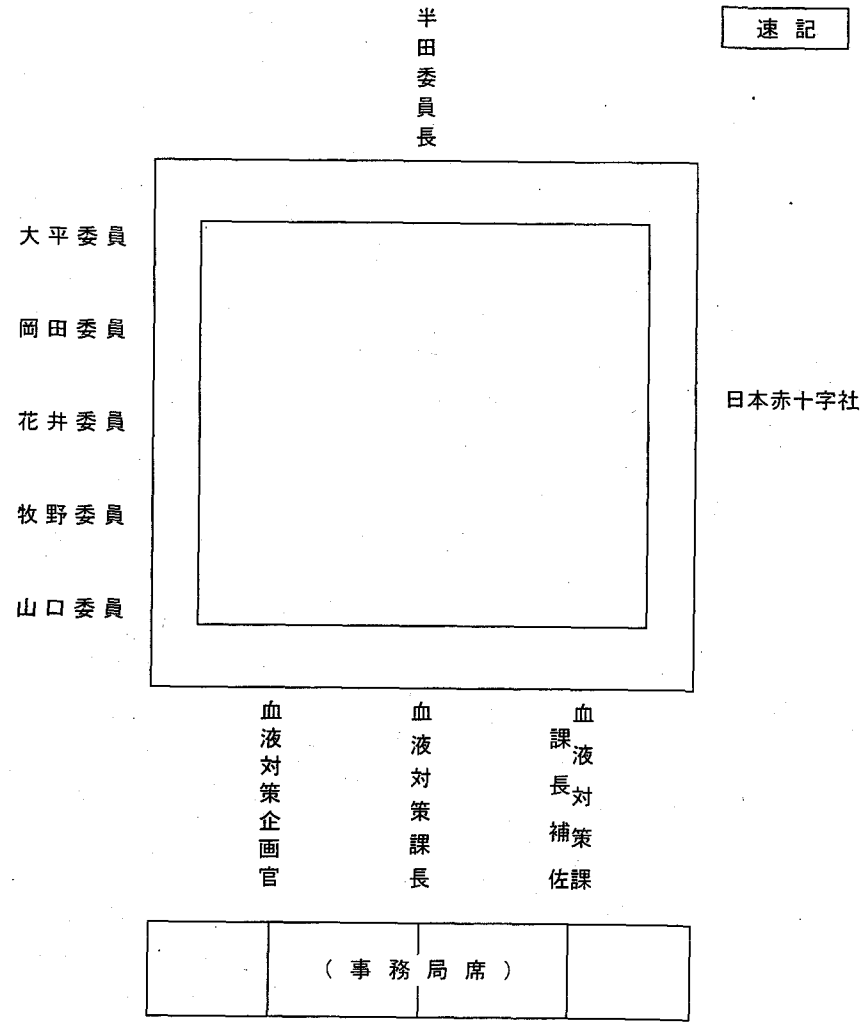
委員名簿

資　料　１　平成２３年度第３回血液事業部会運営委員会議事要旨（案）
資　料　２　感染症定期報告について
資料３－１　供血者からの遡及調査の進捗状況について
資料３－２　血液製剤に関する報告事項について
資料３－３　献血件数及びＨＩＶ抗体・核酸増幅検査陽性件数
資　料　４　ＸＭＲＶに関する文献報告
資　料　５　フィブリノゲン製剤納入先医療機関の追加調査について
追加資料１　血小板製剤に対する感染性因子低減化（不活化）技術の導入準備について（追加報告）（日本赤十字社提出資料）

平成２３年度第４回 薬事・食品衛生審議会薬事分科会 血液事業部会運営委員会 座席表

平成24年3月14日(水)
厚生労働省 12階
専用第15・16会議室
14:00 ～ 16:00

速記



傍聴席

薬事・食品衛生審議会薬事分科会
血液事業部会運営委員会 委員名簿

1．大平 勝美 （おおひら　かつみ）
　　はばたき福祉事業団理事長

2．岡田 義昭 （おかだ　よしあき）
　　国立感染症研究所血液・安全性研究部第一室長

3．花井 十伍 （はない　じゅうご）
　　ネットワーク医療と人権理事

4．半田 誠 （はんだ　まこと）
　　慶應義塾大学医学部輸血・細胞療法部教授

5．牧野 茂義 （まきの　しげよし）
　　国家公務員共済組合連合会虎の門病院輸血部長

6．山口 照英 （やまぐち　てるひで）
　　国立医薬品食品衛生研究所生物薬品部研究員

（50音順、敬称略）

薬事分科会血液事業部会運営委員会規程

（目的）

第１条　この規程は薬事分科会規程（以下「規程」という。）第２条第１項に基づき設置される血液事業部会（以下「部会」という。）に置かれる運営委員会（以下「委員会」という。）の運営に関し必要な事項を定め、審議の円滑な実施を図ることを目的とする。

（所掌）

第２条　委員会は、規程第３条第５項に規定する部会が調査審議すべき血液製剤（血液製剤代替医薬品を含む。以下同じ。）に係る事項（以下「部会で調査審議すべき事項」という。）を検討するとともに、以下に掲げる事項を確認し、部会に報告するものとする。

一　安全な血液製剤の安定供給の確保等に関する法律（以下「血液法」という。）第２６条第１項に規定する血液製剤の製造又は輸入の実績に係る報告

二　血液法第２９条に規定する薬事法第６８条の８第１項に規定する生物由来製品（血液製剤に限る。）の評価に係る報告

三　規程第４条第１項の規定に基づき部会に置かれる調査会における調査審議の状況

四　その他部会で調査審議する事項のうち特別の事項についての状況

（委員会への所属）

第３条　委員会に所属すべき委員は、部会に所属する委員、臨時委員及び専門委員（以下「委員等」という。）の中から、部会長が指名する。

２　部会長は、前項の規定により委員会に属すべき委員等を指名する場合は、血液製剤を使用する患者の代表、医療関係者、血液事業の専門家を含め、数名を指名する。

３　部会長は、第一項の規定により委員会に属すべき委員等を指名した場合は、部会においてその旨を報告しなければならない。

（委員長の選任）

第４条　委員会に委員長を置き、委員会に属する委員等の互選により選任する。

２　委員長は、委員会の事務を掌理する。

３　委員長に事故があるときは、委員会に属する委員等のうちから委員長があ

らかじめ指名する者が、その職務を代理する。

（委員会の開催）

第５条　委員会は、四半期（１月から３月まで、４月から６月まで、７月から９月まで及び１０月から１２月までの各期間をいう。）ごとに開催する。

２　前項に規定する場合のほか、委員等が必要と認めるときは委員会を開催することができる。

（議決）

第６条　部会への報告の要否等、議決を行う必要がある委員会の議事は、委員会に属する委員等で会議に出席したものの過半数で決し、可否同数のときは、委員長の決するところによる。

（議事の公開）

第７条　委員会は原則として公開する。ただし、公開することにより、委員の自由な発言が制限され公正かつ中立な審議に著しい支障をおよぼすおそれがある場合、又は、個人の秘密、企業の知的財産等が開示され特定の者に不当な利益又は不利益をもたらすおそれがある場合については、委員長は、これを非公開とすることができる。

（雑則）

第８条　この規程に定めるもののほか、委員会の運営に関し必要な事項は、部会長が部会に諮り決定するものとする。

附則

この規程は、平成１５年７月３０日から施行する。

資料１

平成23年度第3回　血液事業部会運営委員会議事要旨（案）

日時：　平成23年12月13日（火）　16:00～18:00
場所：　厚生労働省19階　専用第23会議室
出席者：

（委員）

半田委員長、大平、岡田、花井、牧野、山口各委員

（採血事業者）

日本赤十字社血液事業本部　田所経営会議委員、日野副本部長、石川血漿分画事業統合推進室主幹、五十嵐臨床開発課長

（事務局）

三宅血液対策課長、丈達血液対策企画官、伯野課長補佐

議　題：
1. 議事要旨の確認
2. 感染症定期報告について
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4. 日本赤十字社からの報告事項について
5. その他

（審議概要）

<u>議題1について</u>

議事要旨に関する意見等については、事務局まで連絡することとされた。

<u>議題2について</u>

感染症定期報告について、事務局から説明後、質疑応答がなされた。

<u>議題3について</u>

事務局及び日赤から、供血者からの遡及調査の進捗状況、血液製剤に関する報告事項、献血件数及びHIV抗体・核酸増幅検査陽性件数について説明後、下記のような議論がなされた。

（血液製剤に関する報告事項）

事務局より、セラチア菌（*Serratia liquefaciens*）の感染疑い事例（死亡例）について報告がなされた。委員より、使用後バッグが残されておらず感染源の特定は困難であるが、保存期間や保存条件等から因果関係は否定的ではないかとの意見が出された。また、製剤の外観チェックや使用バッグの保存等の対応については、輸血療法の実施に関する指針や血液製剤等に係る遡及調査ガイドラインに基づき、引き続き、医療機関に対して周知を図ることが必要との意見が出された。

（献血件数及び HIV 抗体・核酸増幅検査陽性件数）

委員より、献血者における検査陽性件数と、都道府県ごとの人口あたりの検査件数を比較することで、検査目的で献血に流れている可能性の有無が検討できるのではないかとの意見が出された。

議題4について

（血小板製剤の不活化技術）

日赤より、血小板製剤に対する不活化技術（MIRASOL）導入の準備状況について報告がなされた。委員より、共同開発企業を介する等して、より積極的に情報収集に努める必要があるとの意見が出された。

（日赤と田辺三菱の血漿分画事業統合の進捗状況）

日赤より、田辺三菱との血漿分画事業の統合に関する進捗状況について報告がなされた。東日本大震災の影響により両社間の検討開始が大幅に遅延したため、統合時期は当初の予定から6ヶ月延期し、平成24年10月となることが報告された。

議題5について

（XMRV 関係）

岡田委員より、XMRV に関する最新文献の報告がなされた。いずれの文献も血液を介したXMRV感染の事実を否定するものであったが、事務局においては、引き続き、情報収集に努めることとされた。

（フィブリノゲン関係）

事務局より、フィブリノゲン製剤の調査に係る公表事項の報告がなされた。

以上

資料2

感染症定期報告に関する今後の対応について

平成１６年度第５回
運営委員会確認事項
（平成１６年９月１７日）

1　基本的な方針

運営委員会に報告する資料においては、

(1) 文献報告は、同一報告に由来するものの重複を廃した一覧表を作成すること。

(2) 8月の運営委員会において、国内の輸血及び血漿分画製剤の使用した個別症例の感染症発生報告は、定期的にまとめた「感染症報告事例のまとめ」を運営委員会に提出する取り扱いとされた。これにより、感染症定期報告に添付される過去の感染症発生症例報告よりも、直近の「感染症報告事例のまとめ」を主として利用することとすること。

2　具体的な方法

(1) 感染症定期報告の内容は、原則、すべて運営委員会委員に送付することとするが、次の資料概要を作成し、委員の資料の確認を効率的かつ効果的に行うことができるようにする。

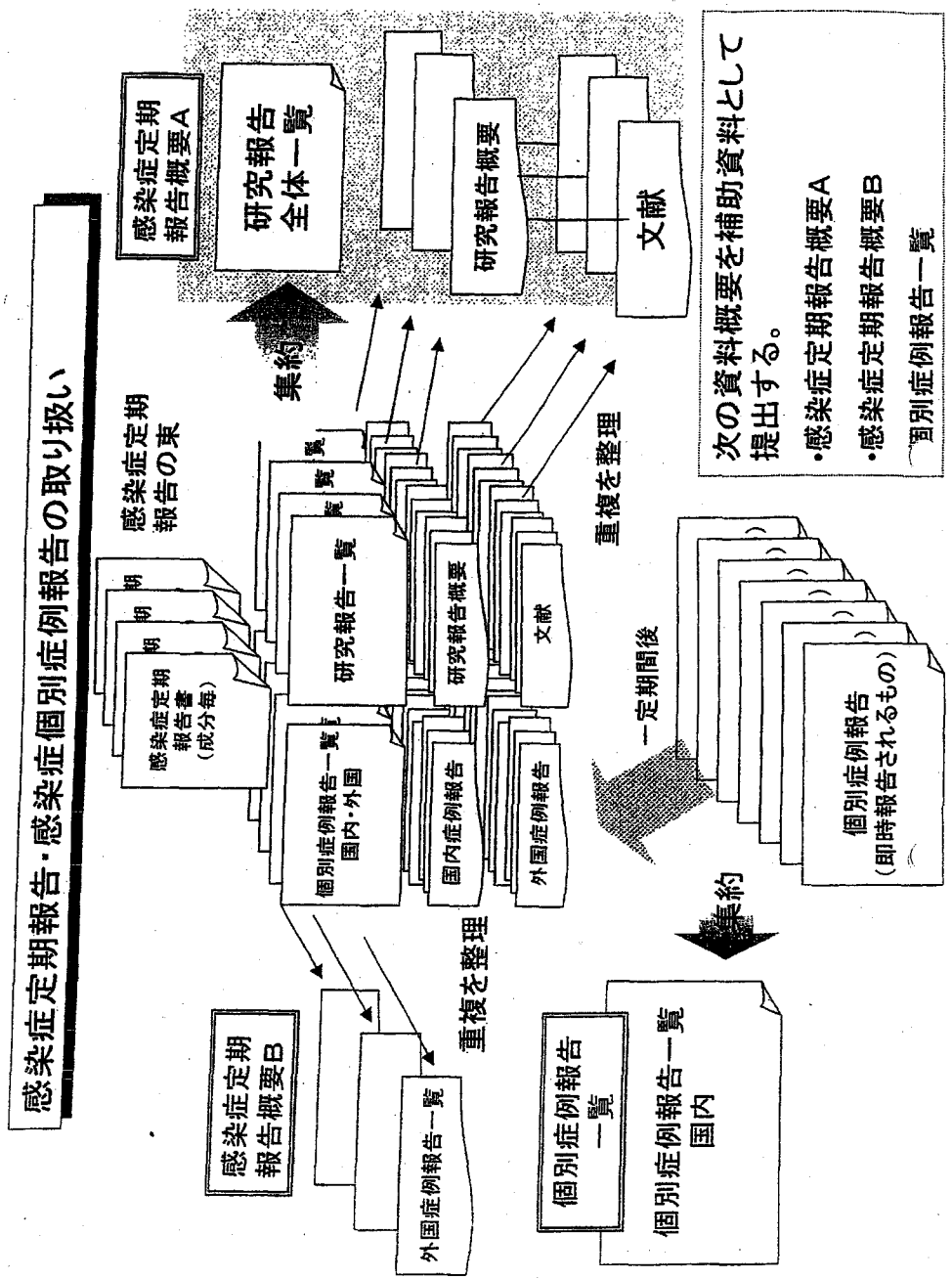
① 研究報告は、同一文献による重複を廃した別紙のような形式の一覧表を作成し、当該一覧表に代表的なものの報告様式（別紙様式第2）及び該当文献を添付した「資料概要A」を事務局が作成し、送付する。

② 感染症発生症例報告のうち、発現国が「外国」の血漿分画製剤の使用による症例は、同一製品毎に報告期間を代表する感染症発生症例一覧（別紙様式第4）をまとめた「資料概要B」を事務局が作成し、送付する。

③ 感染症発生症例報告のうち、発現国が「国内」の輸血による症例及び血漿分画製剤の使用による感染症症例については、「感染症報告事例のまとめ」を提出することから、当該症例にかかる「資料概要」は作成しないこととする。ただし、運営委員会委員から特段の議論が必要との指摘がなされたものについては、別途事務局が資料を作成する。

(2) 発現国が「外国」の感染症発生症例報告については、国内で使用しているロットと関係がないもの、使用時期が相当程度古いもの、因果関係についての詳細情報の入手が困難であるものが多く、必ずしも緊急性が高くないと考えられるものも少なくない。また、国内症例に比べて個別症例を分析・評価することが難しいものが多いため、緊急性があると考えられるものを除き、その安全対策への利用については、引き続き、検討を行う。

(3) 資料概要A及びBについては、平成16年9月の運営委員会から試験的に作成し、以後「感染症的報告について（目次）」資料は廃止することとする。



感染症定期報告概要

（平成２４年３月１４日）

平成２３年１０月１日受理分以降

Ａ　研究報告概要
Ｂ　個別症例報告概要

Ａ　研究報告概要

○　一覧表（感染症種類毎）
○　感染症毎の主要研究報告概要
○　研究報告写

研究報告のまとめ方について

１　平成２３年１０月１日以降に報告された感染症定期報告に含まれる研究報告（論文等）について、重複している分を除いた報告概要一覧表を作成した。

２　一覧表においては、前回の運営委員会において報告したもの以降の研究報告について、一覧表の後に当該感染症の主要研究報告の内容を添付した。

感染症定期報告の報告状況(2011/10/1～2012/1/31)

血対ID	受理日	番号	感染症(PT)	出典	概要	新出文献番号
100466	14-Dec-11	110740	B型肝炎	J Clin Virol. 52(2011)151-154	B型肝炎ウイルス(HBV)ワクチンが無効であった慢性B型肝炎感染の症例報告。2006年4月、同性間性交渉を持つ男性に対してHBVワクチン接種が実施され、ワクチンの増量の後2007年11月には抗HBs抗体が2社の検査法で161mIU/mL及び62mIU/mLになった。2009年12月、患者が疲労感と筋肉痛のため受診したところ、ALT上昇が見られ、検査により慢性HBV感染症であることが確認された。系統発生解析によりこのウイルスはHBVジェノタイプFに一致した。これは、HBVワクチンを接種され抗HBs抗体価が10mIU/mLを上回った者が、HBVジェノタイプFに感染し、慢性化した初めての報告である。	1
100494	30-Jan-12	110879	B型肝炎	http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/default.htm	米国FDAによる、血液製剤のB型肝炎ウイルス(HBV)」感染リスクを低減させるための核酸検査に関するガイダンス案。血液事業者に対し主に以下の事項が勧告されている。 ・人全血液及び血液成分については、HBsAg及びHBcAb陰性の検体に対しHBV NATを実施し、検出限界は100 IU/mLとする。原料血漿についてはHBsAgが陰性の検体に対しHBV NATを実施し、検出限界は500 IU/mLとする。 ・他の感染症関連検査がすべて陰性であれば、輸血用人全血液、血液成分及び原料白血球については、HBV NAT、HBsAg、HBcAbが陰性であること、原料血漿及び回収血漿についてはHBV NAT及びHBsAgが陰性であることをもって、供給を可とする。 ・.NAT結果が陽性の場合、自己血を除いて、供給してはならない。永久供血延期または無期限供血延期とする。 ・供血者の適格性再確認には、少なくとも陽性となった採血から6カ月後にHBsAg、HBcAb及びHBV NATを行い、その結果により、永久的供血延期かリエントリー可能か評価する。	2
100434	24-Oct-11	110642	B型肝炎 C型肝炎	Interviorology. 54(2011)185-195	日本におけるC型肝炎ウイルス(HCV)、B型肝炎ウイルス(HBV)の診断未確定キャリア総数に関する報告。地域及び年齢別のHCVならびにHBV有病率を、20-39歳については初回供血者、40-74歳については定期健康診断の受診者にて調査された。5-19歳の若者の有病率は一つの県において決定され、75歳以上の高齢者のHCV有病率は指数モデルにて推定された。その結果、2005年の127,285,653人の全人口のうち、0.63%がHCVキャリアであると算出され、0.71%がHBVキャリアであると算出された。C型肝炎の年齢による特徴として、年齢とともに有病率の変動幅が大きかった。地域別の特徴として、有病率の変動幅が大きく異なり、西日本で増幅が大きい傾向があった。一方B型肝炎では、全地域において55～59歳で最も有病率が高く、北海道では3.1%に上った。どの地域においても2000年と2005年を比較すると、HCV及びHBVキャリアの割合は減少している。	3
100432	24-Dec-11	110639	E型肝炎	Vox Sanguinis. (2011)1-2	血漿分画プールにおけるE型肝炎ウイルス(HEV)の地理的分布に関する報告。欧州、北米、中東及びアジアから血漿分画プール75例を入手し、HEV RNAと抗HEV IgGの検出を行った。その結果、約10%の血漿分画プールがHEV RNA陽性で、陽性プールの地理的分布は欧州、北米、アジアと広範囲にわたっていたが、いずれも1000copies/mlを超えていなかった。同定された株の系統発生解析により、genotype4がアジアのプールに限定されていたのに対し、genotype3は欧州と北米のプールで確認された。また、抗HEV IgGの濃度はアジアのプールで最も高かった。	4
100472	26-Dec-11	110773	E型肝炎	Vox Sanguinis. Sep 29, 2011	英国における献血者プール血漿中のE型肝炎ウイルス(HEV)RNAの検出に関する報告。血液供給へのHEV感染リスクを調査するために、英国において収集された880例の血漿ミニプールを対象に血清学的及び分子学的調査が行われた。それぞれのプールは48人分の献血から構成されていた。検査の結果、6例(0.7%)のプールにおいてHEV-RNA陽性であり、この6例は全て抗HEV抗体陽性であった。また、HEV-RNA陰性プールのうち100例を検査したところ、73%がHEV IgG陽性であったが、HEV IgM陽性のプールはなかった。これらの結果は血液製剤のHEV感染リスクの可能性を示したものだが、輸血後のHEV感染の範囲については十分な調査がなされていないため、HEVと血液製剤の安全性については更なる調査が必要である。	5

血対ID	受理日	番号	感染症(PT)	出典	概要	新出文献番号
100472	26-Dec-11	110773	パルボウイルス	Transfusion. 51(2011)1896-1908	パルボウイルスB19(B1V9)の血液分画における分布及び持続性に関する報告。パルボウイルスに対するレセプターが赤血球膜上にあることを踏まえて、B19Vの血中分布を調査するため、B19Vをスパイクした血液及びB19V感染ドナーから収集された血液を用いてウイルスDNAの血液分画中の分布を調査した。ウイルススパイク実験では、血液を超遠心分離法によって分画とし、PCRによりウイルスDNAを定量したところ、DNAの約3分の1は血漿中で回収され、3分の2は赤血球に結合していた。また、血中B19V-DNA濃度が100IU/mL以上でIgM陽性期の感染ドナーにおいて、全血と血漿中のウイルスDNA量を比較したところ、DNA濃度の中央値は血漿中よりも全血中で約30倍高かった。一方で、血中のウイルス濃度が低く、IgM陰性時のドナーでは、全血対血漿比は約1であった。これらの結果より、血漿に対する全血のB19V-DNAの比は、ウイルス量低下とIgM反応性低下を伴って減少することが明らかとなった。	6
100472	26-Dec-11	110773	パルボウイルス	Vox Sanguinis. Jul 22, 2011	パルボウイルスB19(B19V)の液状加熱における熱感受性に関する報告。B19V遺伝子型2の熱感受性を調査するために、アルブミン、免疫グロブリン、ハプトグロビン、アンチトロンビンの製造における熱処理工程の直前に採取した検体にB19Vをスパイクし、60°C10時間の加熱処理を行いながら感染性を経時的に測定した。また、低pH免疫グロブリン溶液についてもB19Vをスパイクし、室温で14日間の処理を行った。その結果、B19V遺伝子型2はアルブミン及び免疫グロブリンにおいて急速に不活性化され、ハプトグロビンにおいては速度は遅いものの不活性化が確認された。一方でアンチトロンビンにおいては不活性化が限定的であり、10時間の加熱処理では感染性が残存していた。また、低pH免疫グロブリン溶液においては7日後に不活性化が確認され、これらの結果は全て遺伝子型1でのパターンと同様であった。このことから、B19Vの遺伝子型1及び遺伝子型2は、異なる血漿製剤の間で熱感受性が変化することが示された。	7
100472	26-Dec-11	110773	パルボウイルス	Journal of Virological Methods. 178(2011)39-43	パルボウイルスB19(B19V)に対するフィルター処理のウイルス除去能に関する報告。15～19nmのフィルターのウイルス除去能力を調査するために、アンチトロンビン、ハプトグロビン、免疫グロブリンのそれぞれの製剤にB19Vを添加し、フィルター処理後に感染力分析とquantitative定量的(Q)-PCR分析を行った。その結果、全ての検体において、フィルター処理後の検体は感染力が示されなかったが、ウイルスDNAはQ-PCRにより検出可能であった。しかし、15nmフィルター濾過後の溶液においてウイルスゲノムのサイズは約90%が0.5kb未満であることが確認された。この結果より、フィルター処理によるリダクションファクターは遊離のDNAにより過少評価されている可能性が示唆された。	8
100428	03-Oct-11	110556	インフルエンザ	MMWR. 60(2011)1213-1215	アメリカにおけるインフルエンザウイルスの再集合に関する報告。2011年8月、米国においてブタインフルエンザ(H3N2)感染症例が2例報告された。2症例の間に疫学的関連性は特定されていない。2症例のウイルスは過去に特定されたH3N2ウイルスと類似しているが、8つの遺伝子のうち1つ(M遺伝子)が2009年のインフルエンザA(H1N1)ウイルスに由来するものであった。このことから、2症例に感染したウイルスがブタインフルエンザA(H3N2)ウイルスとインフルエンザ(H1N1)ウイルスの再集合体であることが示唆された。	9
100459	28-Nov-11	110717	インフルエンザ	Transfusion. 51(2011)1949-1956	日本における、輸血によるインフルエンザ(パンデミック[H1N1]2009)感染リスクに関する報告。日本赤十字社血液センターでは献血後情報としてパンデミック(H1N1)2009感染疑いのある献血者から得られた血液製剤の供給を中止した。輸血による感染リスクを調査するため、献血後7日以内にパンデミック(H1N1)2009と診断された579人の献血者から得られた計565の血漿製剤と413の赤血球製剤についてリアルタイムRT-PCRを実施したところ、どのサンプルからもウイルスRNAは検出されなかった。輸血によるパンデミック(H1N1)2009の感染リスクは極めて低いと考えられる。	10

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100484	23-Jan-12	110811	レトロウイルス(XMRV)	Sciencexpress. 334(2011)814-817	Blood XMRV Scientific Research Working Group (SRWG)による、異種指向性マウス白血病ウイルス関連ウイルス(XMRV)を含むマウス白血病ウイルス(MLV)と慢性疲労症候群(CFS)に関する報告。以前にXMRV/MLV陽性と報告された被験者15例(うち14例がCFS患者)及び以前にXMRV/MLV陰性と報告された健康ドナー15例から採取した血液検体を用いて、再度XMRV/MLV-DNAの検出、ウイルス複製能及び抗体検出の検査を行った。サンプルをコード化し二重盲検下で9か所の研究所に分配し、検査を行ったところ、2か所の研究所においてXMRV/MLV陽性と判定されたサンプルがあったが、CFS患者とコントロールとの間で陽性率に差はなかった。今回の試験により、XMRV/MLV検査に再現性は確認できず、供血者へのスクリーニング項目として採用する正当な理由はないことが示唆された。	11
100466	14-Dec-11	110740	ニパウイルス	Epidemiol Infect. 138(2010)1630-1636	バングラディシュにおけるニパウイルス(NiV)感染の報告。NiVのヒト-ヒト感染について検討するために、バングラデシュにてNiVに感染した患者1例とその患者と身体的接触のあった知人14例の動向を調査した。その結果、14例のうちNiV感染を発症したのは6例(43%)であった。未感染群と比較して感染群ではNiV患者の咳嗽中に同室に滞在していた割合が高かった。NiVの感染拡大を防ぐためには、患者との身体的接触を最小限とすることが求められる。	12
100432	24-Oct-11	110639	ウイルス感染	Plos Pathogens 7(7); e1002155; 2011	異種間で伝播する新規アデノウイルスに関する報告。カルフォルニア国立霊長類研究所(CNPRC)において、サル(titi monkey)にアウトブレイクを起こした新規アデノウイルス(TMAdV)が特定された。建屋内のサル65例のうち23例が劇症の肺炎に進行する上気道症状と肝炎を発症し、そのうち19例が死亡又は安楽死とした。集団発生時にサルと接触した研究者も急性呼吸器疾患を発症し、回復期に血清中TMAdVが陽性であることが確認された。また、米国西部におけるランダムな成人供血者81例のスクリーニングにおいて2例にTMAdV特異的中和抗体が検出された。TMAdVの発見は、新規アデノウイルスが異種間アウトブレイクの潜在的原因として厳重に監視される必要があることを示している。	13
100442	28-Oct-11	110661	ウイルス感染	CCDR Weekly Infectious Diseases News Brief. July 22,2011	異種間で伝播する新規アデノウイルスに関する報告。カルフォルニア国立霊長類研究所(CNPRC)においてサル(titi monkey)にアウトブレイクを起こした新規アデノウイルス(TMAdV)について、集団発生時にサルと接触した研究者も急性呼吸器疾患を発症した。また、研究者の家族にも同様の症状が2例に認められ、血中TMAdVに対する抗体が陽性であることが確認された。研究者の家族は感染サルへの接触がないことから、このウイルスがヒト間でも感染を引き起こした可能性が示唆された。一方、ヒトからサルに感染した可能性も否定できないと報告されている。	14
100459	28-Nov-11	110717	ウイルス感染	Emerging Infectious Diseases. 17(2011)1417-1420	米国における新種のアレナウイルス感染の報告。米国で急性中枢神経疾患や鑑別不能型熱性疾患の患者1,185例中41例(3.5%)から抗ホワイトウォーターアロヨウイルス(WWAV)抗体又は抗リンパ球性脈絡髄膜炎ウイルス(LCMV)抗体が検出された。ペア血清サンプルの抗体価の分析結果から、ノースアメリカンタカリベセロコンプレックスウイルス(NATSV)が2例、LCMVが3例の疾患原因であると示唆された。この研究結果より、NATSVもLCMVと同様に米国内でヒトの疾患原因となることが明らかとなった。	15
100434	24-Oct-11	110642	セラチア	http://www.adph.org/news/assets/110407.pdf	米国における完全静脈栄養剤(TPN)によるSerratia marcescens (S.marcescens)感染の報告。アラバマ州公衆保健局(ADPH)は2つの病院から、TPNを投与した患者にS.marcescens感染が生じたという報告を受けた。同一業者からTPNを納入していた6つの病院が特定され、19症例が報告された。遺伝子解析の結果、TPNを製造する際に使用していた器具及びTPNから分離された菌と、TPNを受けた入院患者12人から分離されたS.marcescensが同じであったと確認した。さらにTPNの原料である混合アミノ酸1袋も、S.marcescensで汚染されていた。TPNを混合する時の殺菌工程の失敗が、汚染の原因になったと考えられる。この製造業者は通知を受け、汚染の可能性を情報提供し、生産を中止し、製品を回収した。	16

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100434	24-Oct-11	110642	大腸菌性胃腸炎	Eurosurveillance vo.16 Is.24	志賀毒素/ベロ毒素産生大腸菌(STEC/VTEC)感染のアウトブレイク発生時におけるドイツのサーベイランスに関する報告。2011年5月、ドイツのロベルト・コッホ研究所は、STEC/VTEC感染による溶血性尿毒症症候群(HUS)患者の急激な増加を受け、サーベイランスについて以下のような変更を行った。疫学情報交換の集約化/国レベルまでの情報伝達の迅速化/病院の救急部における出血性下痢症の症候群サーベイランスシステムの導入/ドイツにおけるHUS治療受け入れ能力の評価/検査機関でのアクティブサーベイランスの開始。これらの追加サーベイランスシステムは今回のアウトブレイクにおいて、より迅速なモニタリングを可能にし、患者の発生動向等について把握することができた。	17
100432	24-Oct-11	110639	細菌感染	CDC/MMWR. 60(2011)1083-1086	米国におけるレジオネラ症発生状況に関する報告。2000~2009年に国立届出疾病監視システム(NNDSS)に報告されたレジオネラ症(レジオネラ病及びポンティアック熱)症例を評価したところ、米国のレジオネラ症年間報告数は2000年の1,100例から2009年の3,522例へと増加し、粗国内発生率も2000年0.39/10万人から2009年1.15/10万人と増加傾向が確認された。	18
100432	24-Oct-11	110639	細菌感染	N Engl J Med. 365(2011)422-429	米国における新規エーリキア症に関する報告。米国におけるエーリキア症の原因菌について調査するために、患者の血液検体について分子学的方法、培養及び血清学的検査を行った。その結果、ミネソタ州とウィスコンシン州の4症例について、既に知られているE. chaffeensis 、E. ewingiiではない新規のエーリキア種により引き起こされたことが明らかとなった。全ての患者は発熱、倦怠感、頭痛及びリンパ球減少症がみられ、3例は血小板減少症、2例は肝酵素濃度上昇を有していた。全例がドキシサイクリン治療により回復した。また、ミネソタ州とウィスコンシン州で採取された697例のクロアシマダニの少なくとも17例は、同一のエーリキア種がPCR検査陽性であった。遺伝子解析により、この新規エーリキア種がE. murisと近縁種であるしていることが示された。	19
100434	24-Oct-11	110642	細菌感染	21st Regional Congress of the International society of Blood Transfusion; P-384 June18-22,2011	赤血球輸血によるヒト顆粒球アナプラズマ症(HGA)感染に関する報告。36歳女性が帝王切開術を受け、6単位の赤血球と2単位の新鮮凍結血漿が輸血された。9日後に発熱を生じ、後に急性呼吸窮迫症候群(ARDS)に転帰した。PCRによってアナプラズマ・ファゴサイトフィルムが検出され、HGAであることが確認された。感染原因として可能性のあるものは輸血のみであった。輸血された8単位についてPCR及び間接蛍光抗体法により検査を行った結果、1検体が陽性を示した。白血球除去赤血球の輸血により感染が発生したため、白血球除去の効果は小さい可能性が示された。この感染症例は、ダニ咬傷歴のある供血者の一時的な供血停止を支持している。	20
100459	28-Nov-11	110717	感染	IASR. 32(2011)218-219	日本におけるライム病の発生状況に関する報告。1987年に長野で1例目が報告されて以来、日本におけるライム病は主に北海道、本州中部以北で200例以上の確定例の存在が推定される。1995~2000年に北海道で集積したマダニ刺咬症700例のうち確定例が56例(8.0%)であることから、ボレリア汚染地域においてライム病の発症頻度はマダニ刺咬症の10%未満と推定される。また1989~2004年までに113例の確定例を集積した結果、北海道のライム病は皮膚症状が主体で、第II期以後の出現頻度も9例(8.0%)と欧米に比べ低い。また発熱、全身倦怠感などの全身症状の出現頻度も低く、抗菌薬に対する反応も良好で、一般に軽症例が多い。その原因は、ボレリアそのものの病原性の違いや、人種的遺伝的違い、医療状況、マダニの違いなど、複数の要因が関与していると推定される。	21

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100459	28-Nov-11	110717	バベシア症	CDC Media Relations. Sep 6, 2011	米国におけるバベシア症の輸血感染リスクに関する報告。自覚のないバベシア症感染供血者スクリーニングに対してFDA認可のバベシア検査は利用できない。米国のほとんどのダニ媒介バベシア症は7州において(コネチカット州、マサチューセッツ州、ミネソタ州、ニュージャージー州、ニューヨーク州、ロードアイランド州、ウィスコンシン州)特に暖かい時期に発生している。しかし輸血関連バベシア症は19州において認識され、年間を通して発生している。バベシア症はマラリアと誤診されることがあり、診断が考慮されない限り重症例でも見逃されやすいと指摘される。2011年1月、バベシア症は全国的な届出疾患となり、当保健省はバベシア症例に関して米国疾病管理予防センター(CDC)と情報共有することを奨励した。バベシア症に関する正確な情報を得ることは、血液供給をより安全にするために有益である。	22
100434	24-Oct-11	110642	トリパノソーマ症	第59回日本輸血・細胞治療学会総会; 2011.4.14-16; O-120	在日ブラジル人献血者におけるTrypanosoma cruzi(T. cruzi)抗体検査に関する報告。シャーガス病は中南米で流行し、感染者はT.cruziを長期間体内に保有する無症候性のキャリアとなることが知られている。在日ブラジル人の献血希望者20例についてT.cruzi抗体検査を行ったところ、ELISA法は20例全員陰性であったが、迅速法は19例陰性、1例判定保留であった。追加検査の結果、判定保留の1例は偽陽性であると判断した。年齢は20代9例、30代11例であった。出身地はサンパウロ州17例、パラナ州2例、ミナスジェイラス州1例であった。全員家族にシャーガス病の者はおらず、また過去にT.cruzi抗体検査をした者は1名のみであった。	23
100432	24-Oct-11	110639	異型クロイツフェルト・ヤコブ病	FDA TSEAC 23rd Meeting. Aug 1,2011	FDAの伝達性海綿状脳症諮問委員会(TSEAC)における議題要約書。FDAは、サウジアラビアにおけるBSEに感染したと考えられる3症例を受け、サウジアラビアでの滞在期間を血液製剤等のドナーの除外条件とすることについてTSEACに助言を求めている。 議題:1)以下の者について血液製剤、組織・細胞由来製剤(HCT/P)のドナーとして不適とすることについて。A) 1980～1996年に米軍としてサウジアラビアに6ヶ月以上滞在した者。B) 1980～1996年にサウジアラビアに累積5年以上滞在した者。 2)血液製剤、HCT/Pの供給及び安全性への上記事項の影響について。 3)更なる安全性基準の必要性について。	24
100494	30-Jan-12	110879	異型クロイツフェルト・ヤコブ病	Haemophilia. 17(2011)931-937	英国の先天性出血性疾患患者におけるvCJD感染リスクに関する報告。英国血友病センター医師機構(UKHCDO)により、供血後vCJDを発症した8人のドナー由来血漿を含む1987-1999年の25バッチの何れかの血液凝固因子製剤の投与を受けた先天性出血性疾患患者におけるvCJD感染リスクが推定された。787例の患者はプロスペクティブに10-20年間調査され、総vCJD感染性は薬剤の総投与量から推測される累積感染性がら推算された。薬剤の投与を受けてから13年以上追跡調査された604例における推定vCJDリスクは595例が1%以上、164例が50%以上、51例が100%という結果であった。これらのリスクが食事によるリスクに上乗せされる。なお、94例はvCJDを発症した患者由来のバッチを供血後6ヵ月以内に投与されていた。2009年1月1日現在、これらの患者でvCJDを発症した患者はいないことは、血漿画分の感染性が過度に見積もられているか、血球製剤の受血者よりも潜伏期間が長いことが原因であると考えられる。	25

別紙様式第2-1

No. 4

医薬品 研究報告 調査報告書

識別番号・報告回数		報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
			2011. 10. 18	該当なし	

一般的名称	人全血液	研究報告の公表状況	O'Halloran JA, De Gascun CF, Dunford L, Carr MJ, Connell J, Howard R, Hall WW, Lambert JS. J Clin Virol 2011 Oct 52(2) 157-62	公表国	使用上の注意記載状況・その他参考事項等
販売名(企業名)	人全血液-LR「日赤」(日本赤十字社) 照射人全血液-LR「日赤」(日本赤十字社)			アイルランド	人全血液-LR「日赤」 照射人全血液-LR「日赤」 血液を介するウイルス、細菌、原虫等の感染 vCJD等の伝播のリスク

研究報告の概要

○B型肝炎ウイルスワクチンが無効であった慢性B型肝炎感染
2006年4月、男性パートナーと無防備な性交渉を持つ男性が性病検査のためクリニックを訪れた。HIV、HBV、HCV検査は全て陰性であった。Engerix-B B型肝炎ワクチン(GlaxoSmithKline)接種が実施されたが、2007年8月患者の抗HBs抗体価は10mIU/mL未満であった。ワクチン量が増やされ、2カ月後に抗HBs抗体価は13mIU/mLとなった。2007年11月には抗HBs抗体は2社の検査法で161mIU/mL及び62mIU/mLになったため、ワクチンに対して満足な反応があるとみなされた。2009年12月、患者はs抗体価は10mIU/mL未満であり、加えてHBV DNAが1.7×10^{8}IU/mLを上回るレベルで検出された。系統発生解析によりこのウイルスはHBVジェノタイプFに99%(3214/3215-bp)以上一致した。そのサブジェノタイプF1bは以前アルゼンチン北部で検出され、同性愛男性の間に蔓延していると報告されていた。この患者は2007年と2009年にアルゼンチンを訪れていた。患者は最初抗ウイルス治療は受けず、3カ月毎のフォローアップがされ、2011年の血清学的検査でウイルス量が1.7×10^{8}IU/mL、HBe抗原陽性の慢性HBV感染症であることが確認された。
これは、HBVワクチンを接種され抗HBs抗体価が10mIU/mLを上回る血清学的反応を示した者が、HBVジェノタイプFに感染し、慢性化した初めての報告である。この症例は、ワクチン投与が成功に見えたにも関わらず、HBV感染を防ぐ事ができなかったことを示している。英国や米国を含む多くの国において、ワクチン接種後の抗HBs抗体価が10mIU/mL以上であれば効果があるとされ、ワクチン量の増加は推奨されていない。一方、アイルランドなどではより高い抗体価が必要だとしている。

報告企業の意見

HBVワクチンを受け、抗HBs抗体価が10mIU/mLを上回る血清学的反応を示した者がHBV ジェノタイプFに感染し、慢性化した初めての報告である。

今後の対応

今後も引き続き情報の収集に努める。

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Case report

Hepatitis B virus vaccine failure resulting in chronic hepatitis B infection

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1. Case description

A 38 year old asymptomatic male presented to the sexually transmitted infections (STI) clinic in a large teaching hospital in April 2006 for a sexual health screen. He had no significant past medical history although he had engaged in several episodes of unprotected sexual intercourse with male partners in the preceding six months. Serological investigations indicated recent/active *Treponema pallidum* infection for which he was treated. Additional serological investigations for human immunodeficiency virus (HIV), hepatitis B virus (HBV) – comprising hepatitis B surface antigen (HBsAg), antibody to hepatitis B core (anti-HBc) and antibody to surface antigen (anti-HBs) – and hepatitis C virus (HCV) were all negative. A course of Engerix-B® (GlaxoSmithKline) hepatitis B vaccine was commenced and completed at 1 and 6 months following the first dose. In August 2007, the patient's anti-HBs titre was found to be <10 mIU/mL (Table 1). A booster dose was administered and titres measured two months after this were 13 mIU/mL (Abbott Architect). A further booster dose was administered and the anti-HBs titre in November 2007 was 161 mIU/mL and 62 mIU/mL on the Biomerieux VIDAS and Abbott Architect anti-HBs assays respectively. At that time, he was considered to have mounted a satisfactory response to vaccination due to the presence of an anti-HBs titre of >10 mIU/mL in two assays. In December 2009, the patient presented to his primary care physician feeling generally unwell, with non-specific symptoms including fatigue and myalgia. Routine biochemical investigations revealed an elevated alanine aminotransferase (ALT) 211 (5–40) with a normal bilirubin 14 (0–20) and he was referred to the infectious diseases service in a tertiary care hospital for further assessment and management. Serological investigations detected the presence of HBsAg, anti-HBc-specific IgM, and HBeAg, consistent with acute HBV infection (Table 1). Of note, the patient's anti-HBs titre was now <10 mIU/mL. These results were confirmed on a follow-up sample five days later. In addition, HBV DNA (Roche AmpliPrep) was detected at a level of >170 000 000 IU/mL. Serological investigations for HIV and HCV were negative. The patient's entire 3215-bp HBV genome was amplified and sequenced on both strands (Genbank accession number HQ378247).[1] Phylogenetic analysis determined the virus was >99% (3214/3215-bp) similar to a HBV genotype F, sub-genotype F1b previously detected in northern Argentina and a subtype that has previously been reported as circulating in MSM in the country (see Fig. 1).[2,3] The patient had visited Argentina on two occasions in the past between 2007 and 2009. Based on amino acid residues 122K, 160K and 127L of the *S* gene this virus was determined to be the *adw4* serotype.[4] Further analysis of the *S* gene did not reveal the presence of significant vaccine escape mutations such as G145R in the immunodominant 'a' region or any deletions in the *S* gene. The wild-type AGG bases from nucleotides 1762 to 1764 and the wild-type G base at nucleotide position 1896 were observed in the basal core promoter and precore regions of the genome respectively, in keeping with the positive eAg serology.[5] The patient did not receive anti-viral treatment at the time of initial presentation

Abbreviations: Anti HBs antibodies, anti hepatitis B surface antibodies; HBV, hepatitis B virus; STI, sexually transmitted infection; HIV, human immunodeficiency virus; HBsAg, hepatitis B surface antigen; Anti-HBc, hepatitis B core antibody; HCV, hepatitis C virus; ALT, Alanine aminotransferase; HBeAg, hepatitis B e antigen; MSM, men who have sex with men.

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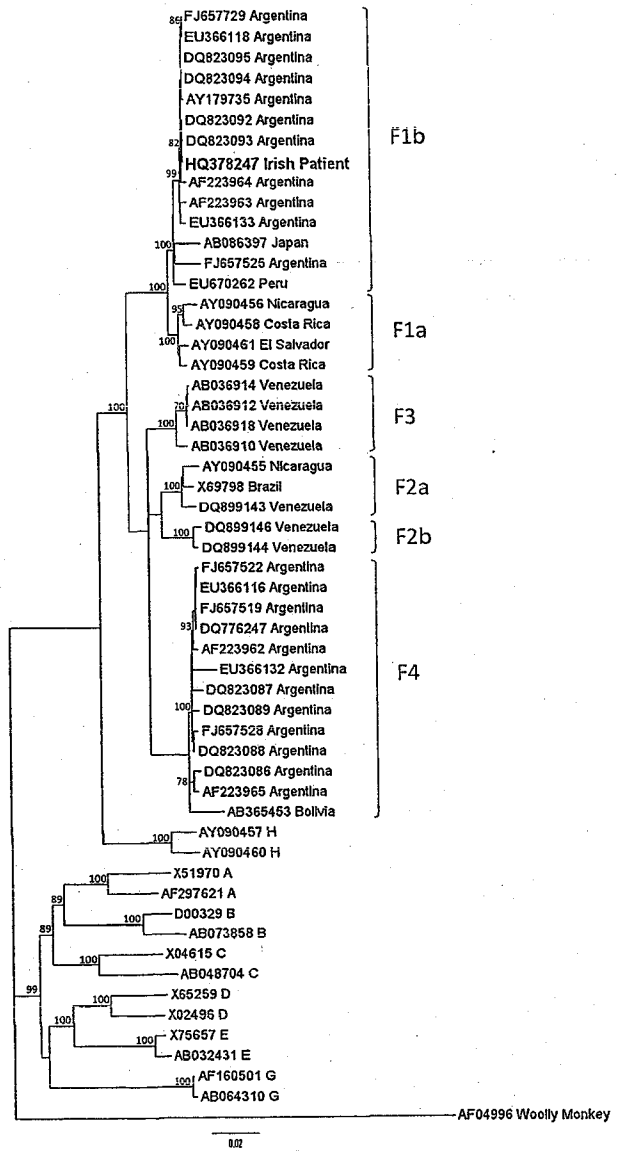

Fig. 1. Neighbour joining phylogenetic tree based on Kimura-two-parameter model on the complete genome of HBV genotypes A–H. Phylogenetic analysis was performed using reference sequences from Genbank, indicated on the tree by their Genbank accession numbers. Woolly Monkey HBV was used as outgroup. Country of origin is included on the nodes of all genotype F reference sequences. The query sequence from this study is represented in boldface. The tree was built in PAUP* (Sinauer Associates, Inc. Publishers). The numbers at the nodes indicate the percentage bootstrap replicates (n = 1000) higher than 70%.

Table 1
Development of Hepatitis B serological markers from April 2006 through August 2010.

Date	Vaccine	Anti-HBs	Status
03/04/2006			Negative: HBsAg
21/04/2006		<10 mIU/mL (architect)	
22/01/2007		13.28 mIU/mL (architect)	Negative: HBsAg
29/08/2007		62.79 mIU/mL (architect)161 mIU/mL (Vidas)	Negative: HBsAg, anti-HBc total
08/10/2007			*Negative: HBsAg, anti-HBc total*
12/11/2007		<10 mIU/mL (architect), 27 mIU/mL (Vidas)	
22/10/2009			Positive weak: HBsAg Negative: HBeAg, HBeAb, anti-HBc total, anti-HBc IgM
17/12/2009		Not detected (architect)	Positive: HBsAg, HBeAg, anti-HBc total, anti-HBc IgM Negative: HBeAb
22/12/2009			*Positive: HBsAg, HBeAg, anti-HBc total, anti-HBc IgM, DNA (>170 000 000 IU/mL)* Negative: HBeAb
09/02/2010		Not detected (architect)	Positive: HBsAg, HBeAg, anti-HBc total, anti-HBc IgM, DNA (98 589 232 IU/mL) Negative: HBeAb
03/03/2010			*Positive: HBsAg, HBeAg, anti-HBc total, anti-HBc IgM* Negative: HBeAb
26/08/2010			Positive: HBsAg, HBeAg DNA (>170 000 000 IU/mL) Negative: HBeAb, anti-HBc IgM

and was followed up at 3 monthly intervals. Subsequent serological investigation in June 2011 confirmed HBeAg-positive chronic HBV infection with a viral load of >170 000 000 IU/mL.

2. *Why this case is important*

This report describes, what is to the authors' knowledge, the first case of HBeAg-positive chronic HBV infection (genotype F) occurring in an individual who received several doses of HBV vaccine and demonstrated an anti-HBs serological response of >10 mIU/mL, the accepted threshold for protection.[6,7] This finding suggests this patient was not protected from chronic HBV despite seemingly successful vaccination. There is ongoing debate regarding the post vaccine anti-HBs response necessary to protect against HBV infection. Many countries (including the United Kingdom and the United States) use a value of >10 mIU/mL as a threshold to determine immunity and do not routinely recommend booster doses of vaccine, whereas others (including Ireland) require higher levels (>100 mIU/mL) of anti-HBs to indicate protection.[8] While the duration of long term immunity post vaccination is unclear, recent meta-analyses suggests it lasts for up to twenty years in immune competent patients.[9–13]

3. *Other similar and contrasting cases in the literature*

Primary HBV vaccine failure is not uncommon among adults[14] with studies estimating that up to 10% of immune competent adults do not respond to HBV vaccine.[15,16] However, very few cases of vaccine failure have been documented in immune competent individuals who have an adequate anti-HBs response.[17,18] A previous report described acute HBV genotype A infection 14 years post successful vaccination,[17] although arguably this report does not constitute vaccine failure. On the contrary, the report of Boot and colleagues actually serves to demonstrate the long-term effectiveness of vaccination in protecting against chronic HBV infection.[17] Similarly, acute HBV genotype F has previously been reported in a successfully vaccinated (Twinrix®) immune competent German patient.[18] Importantly, no HBsAg escape mutations were detected, and the infection resolved.[18] It is known that in highly vaccinated populations, individuals vaccinated against HBV may become infected. However, they do not typically progress to chronic HBV infection even in the setting of waning anti-HBs titres, as immunologic memory confers sufficient immunity.[19] Nevertheless, in the case reported herein, two years following seemingly successful vaccination, a young homosexual male presented with acute genotype F HBV infection that progressed into chronicity. It is unclear why this patient should have developed chronic HBV infection, although acknowledged the final anti-HBs levels remained relatively low despite five doses of vaccine.

4. Discussion

Current vaccination for HBV utilises a recombinant antigen targeting the immunodominant 'a' determinant region of the S gene of HBV. The Engerix-B® vaccine used in this case employs the recombinant small envelope S protein of HBV genotypes A and D, the most common circulating genotypes in Ireland, Europe and North America. However, sequence differences exist in this region between HBV genotype F viruses and genotypes A and D.[18] Indeed, genotype F is one of the most genetically diverse HBV genotypes. It is found predominantly in Central and South America[20] and rarely in Ireland[21] (unpublished data). The detection of a genotype F strain is in keeping with the sexual history of the present case, who reported multiple unprotected sexual encounters in Argentina. HBV Genotype F also has been associated with acute (typically HBeAg-positive) symptomatic infections in Argentina,[22] and therefore higher viral loads. Thus, it could be suggested that repeated MSM sexual exposure to this genotype and the high viral load encountered by the patient may have overwhelmed any protection conferred by the sub-optimal anti-HBs level. Nevertheless, Pezzano and co-workers reported that genotype F was more commonly associated with acute than chronic infection and therefore the current case is surprising.[22] One potential alternative explanation for the case described is that the initial anti-HBs results were incorrect. This is unlikely. Firstly, testing was performed in an accredited laboratory using internal quality controls to monitor assay performance; in addition, external quality assurance distributions tested over the same time period achieved 100% concordance with expected results. Secondly, despite minor inter-assay variation in the absolute anti-HBs titres obtained, the Architect and Vidas assays each confirmed the results of the other. Thirdly and finally, 3 sequential samples were tested for anti-HBs over a 4 month period, all generating results compatible with the patient's vaccination history. Following these initial results, a decline in anti-HBs over the subsequent 24 months was noted. This decline is not exceptional as levels of vaccine-induced antibody are known to wane over time.[23] Indeed, post-vaccine screening is typically performed 2–4 months following completion of the

course of vaccine to ensure accurate anti-HBs measurement before waning ensues. To the authors' knowledge, this is the first report of a patient developing chronic HBV infection with a genotype F virus following an apparently adequate anti-HBs response. The patient was otherwise healthy and immune competent; no known escape mutations were identified in the viral genome; and it is established that the HBV vaccine protects against chronic infection in the immunocompetent individual. However, this case illustrates a scenario in which the level of protection conferred from a sub-optimal vaccine-induced immune response may not protect against significant challenge with a high viral load heterotypic HBV genotype infection. Therefore it may be necessary to review the protective level in those groups, such as MSM, who may be at increased risk of exposure to HBV infected individuals with high viral loads.

Funding

None.

Competing interest

None declared.

Ethical approval

Not required.

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医薬品
医薬部外品　　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2011 年 11 月 30 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	人ハプトグロビン		研究報告の公表状況	fda.gov/BiologicsBloodVaccines/NewsEvents/WhatsNewforBiologics/2011/11/29	公表国 アメリカ	
販売名 (企業名)	ハプトグロビン静注 2000 単位「ベネシス」　(ベネシス)					

-15- 研究報告の概要

業界のためのガイダンス
― 全血および血液成分(原料血漿を含む)のドナーから得られた個々のサンプルおよびそれらのプールについての、B 型肝炎ウイルス伝播危険性を低減させるための核酸検査の使用について ―

ガイダンス案

FDA(米国食品医薬品庁)は、輸注またはそれを原料として製造するために全血または血液成分(それには、リカバード血漿、原料血漿、および原料白血球を含むが)を採取する血液採取・取扱施設に対する、FDA 認可済の核酸増幅検査(NAT)を B 型肝炎ウイルス(HBV)デオキシリボ核酸(DNA)の有無について血液ドナーをスクリーニングすることに関しての勧告事項をここに提示する。また、FDA は製品試験とその処置、ドナー管理、ドナーの再適格化、および製品の表示についての勧告事項も、血液採取・取扱施設に提示する。

(中　略)

IV. 勧告事項
A. HBV NAT を用いたドナースクリーニング
§610.40(b)に従って、血液採取・取扱施設は FDA がスクリーニング検査用として認可したスクリーニング法を、製造者の使用説明書に従って用いなければならない。血液採取・取扱施設は、HBV を含む伝染性疾患の伝播の危険性を十分かつ適切に低減させるために必要に応じてそのような検査を 1 種類以上行わなければならない。

1. 輸注用の全血および血液成分、ならびに製造用の原料白血球についての§610.40(b)に規定されている要求事項に合致させるために、FDA は血液採取・取扱施設が、HBsAg と抗 HBc の検出に加えて、FDA が認可した NAT による HBV DNA についてのドナースクリーニング試験を用いることを勧告する。HBsAg および抗 HBc の検出用として FDA が認可した検査法で陰性もしくは無反応であった場合には、FDA は血液採取・取扱施設が、個々のドネーション中の HBV DNA 検出用として検出下限が＜100 IU/mL HBV DNA であるような FDA が認可済の HBV NAT を用いてドネーションをさらに試験することを勧告する。血液採取・取扱施設の用いる FDA 認可済の HBV NAT によるスクリーニングは、ミニプールを用いたドネーションサンプル試験フォーマットで行うことができ、または個々のドネーションを検査するフォーマットで行うこともでき、他のもの、例えば HIV や HCV の検査とともに多重 NAT とすることができ、または HBV のみを検査する NAT とすることができる。HBsAg、抗 HBc、および HBV DNA の NAT による検査は同時に行うことができる。

2. 血漿分画製剤への製造用の原料血漿の検査について§610.40(b)の要求事項に合致させるために、FDA は血液採取・取扱施設が HBsAg 検出用に FDA が認可済のドナースクリーニング検査を用いることを勧告する。HBsAg 検出用の FDA 認可済検査

使用上の注意記載状況・その他参考事項等

2. 重要な基本的注意
(1)本剤の原材料となる献血者の血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体、抗HTLV-1抗体陰性で、かつALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1, HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohnの低温エタノール分画で得た画分から人ハプトグロビンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びウイルス除去膜によるろ過処理を施しているが、投与に際しては、次の点に十分注意すること。

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法で検査して陰性または無反応の場合には、FDA は血液採取・取扱施設が当該ドネーションをさらに、個々のドネーション中の HBV DNA 検出用として検出下限が＜500 IU/mL HBV DNA であるような FDA が認可済の HBV NAT を用いて検査することを勧告する。血液採取・取扱施設の用いる FDA 認可済の HBV NAT によるスクリーニングは、ミニプールを用いたドネーションサンプル試験フォーマットで行うことができ、または個々のドネーションを検査するフォーマットで行うこともでき、他のもの、例えば HIV 検査や HCV 検査とともに多重 NAT とすることができ、または HBV のみを検査する NAT とすることができる。HBsAg および HBV DNA の NAT による検査は同時に行うことができる。(FDA は現在のところ、原料血漿のドナーに抗 HBc 検査を行うことは勧告していない(参照文献 2))。

(後　略)

報告企業の意見	今後の対応
米国で全血および血液成分について HBV NAT を導入するとの FDA のガイダンス（案）である。弊社血漿分画製剤の原料血漿については、以前より HBV のミニプール NAT を導入している。 HBV は直径 42nm の球形をした DNA ウイルスで、ウイルス粒子は二重構造をしており、ウイルス DNA をヌクレオカプシドが包む直径約 27nm のコア粒子と、これを被うエンベロープから成り立っている。万一、原料血漿に HBV が混入したとしても、BVD 及び BHV をモデルウイルスとしたウイルスクリアランス試験成績から、本剤の製造工程において不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

Guidance for Industry

Use of Nucleic Acid Tests on Pooled and Individual Samples from Donors of Whole Blood and Blood Components, including Source Plasma, to Reduce the Risk of Transmission of Hepatitis B Virus

DRAFT GUIDANCE

This guidance is for comment purposes only.

Submit one set of either electronic or written comments on this draft guidance by the date provided in the *Federal Register* notice announcing the availability of the draft guidance. Submit electronic comments to http://www.regulations.gov. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. You should identify all comments with the docket number listed in the notice of availability that publishes in the *Federal Register*.

Additional copies of this guidance are available from the Office of Communication, Outreach and Development (OCOD) (HFM-40), 1401 Rockville Pike, Suite 200N, Rockville, MD 20852-1448, or by calling 1-800-835-4709 or 301-827-1800, or e-mail ocod@fda.hhs.gov, or from the Internet at http://www.regulations.gov, http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.

For questions on the content of this guidance, contact OCOD at the phone numbers or e-mail address listed above.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
November 2011

Table of Contents

Guidance for Industry

Use of Nucleic Acid Tests on Pooled and Individual Samples from Donors of Whole Blood and Blood Components, including Source Plasma, to Reduce the Risk of Transmission of Hepatitis B Virus

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

We, FDA, are providing you, blood establishments that collect Whole Blood and blood components for transfusion or for further manufacture, including recovered plasma, Source Plasma and Source Leukocytes; with recommendations concerning the use of FDA-licensed nucleic acid tests (NAT) to screen blood donors for hepatitis B virus (HBV) deoxyribonucleic acid (DNA). We are also providing you with recommendations for product testing and disposition, donor management, methods for donor requalification, and product labeling.

In addition, we are notifying you in this guidance that we consider the use of an FDA-licensed HBV NAT to be necessary to reduce adequately and appropriately the risk of transmission of HBV. FDA-licensed HBV NAT can detect evidence of infection at an earlier stage than is possible using previously approved hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen (anti-HBc) tests. Therefore, we recommend that you use FDA-licensed HBV NAT, in accordance with the requirements under Title 21 Code of Federal Regulations, 610.40(a) and (b), (21 CFR 610.40(a) and (b)).

This guidance supplements previous memoranda and guidance from FDA to blood establishments concerning the testing of donations for HBsAg and anti-HBc, and the management of donors and units mentioned in those documents (Refs. 1 through 5). Note that testing Whole Blood and blood components for transfusion and Source Leukocytes for further manufacture for HBsAg and anti-HBc, and Source Plasma for HBsAg should continue when a blood establishment implements HBV NAT. [1] FDA may consider advancements in technology

[1] FDA does not currently recommend that Source Plasma donors be tested for anti-HBc. If anti-HBc reactive units were excluded from pools used for the manufacture of plasma derivatives, titers of neutralizing antibody to hepatitis B surface antigen (anti-HBs) in those pools would be expected to diminish, as both these antibodies usually occur together. The presence of neutralizing anti-HBs is believed to contribute to the safety of certain plasma products. (Ref. 2). Plasma units that are untested, non-reactive (NR), or repeat reactive (RR) for anti-HBc are currently acceptable for the manufacture of plasma derivatives (Ref. 2). Consistent with § 610.40(h)(2)(v), recovered plasma

for testing blood donations, as well as data obtained following the implementation of HBV NAT, to make future recommendations on adequate and appropriate testing for HBV.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

II. DEFINITIONS

Discriminatory NAT: A NAT that uses specific primers for HIV-1 or HBV or HCV to identify the RNA or DNA in the reactive multiplex NAT sample as HIV-1 RNA or HBV DNA or HCV RNA. Performing a Discriminatory NAT on a reactive sample is a required step for those establishments using an approved multiplex test. The labeling for licensed multiplex NATs specifies that Discriminatory NAT is to be performed. Under § 610.40(b) (21 CFR 610.40(b)), you must use FDA-approved screening tests in accordance with the manufacturer's instructions.

Donor Reentry: A procedure that qualifies a deferred donor as eligible to donate again. Donor reentry procedures may be used following a false positive test result and typically require the passage of time to allow for possible seroconversion prior to the performance of additional serologic testing and NAT.

HBV NAT assay with a limited supplemental test indication: Some HBV NAT assays have received a limited supplemental indication for repeatedly reactive HBsAg test results. If a donation tests HBV NAT-positive for HBV DNA using an HBV NAT with such a limited supplemental test indication, and if that donation also tests HBsAg repeatedly reactive in a screening test, the HBsAg test result can be recorded as HBsAg positive. In this case, an HBsAg neutralization test need not be performed. However, if a donation tests HBV NAT-negative for HBV DNA using an HBV NAT with such a limited supplemental test indication, and if that donation tests HBsAg repeatedly reactive in a screening test, an HBsAg neutralization test should be performed. In this case, the result of the neutralization test serves as the test of record. (Ref. 1)

Minipool: A pool of donor samples on which NAT (minipool NAT or MP-NAT) is performed as a screening test. A minipool is formed by pooling of samples from subpools or by directly pooling samples from individual donors.

Multiplex NAT: A NAT that simultaneously detects HIV-1 RNA, HBV DNA, and HCV RNA.

Single Virus NAT: A NAT that separately detects either HIV-1 RNA or HBV DNA or HCV RNA.

from donations of Whole Blood that test anti-HBc reactive may be used for further manufacture into plasma derivatives.

III. BACKGROUND

Under § 610.40(a), establishments that collect blood or blood components must test each donation of human blood or blood component intended for use in preparing a product, including donations intended as a component of, or used to prepare a medical device, for evidence of infection due to certain communicable disease agents, including HBV. In addition, under § 610.40(b), you must perform one or more such tests as necessary to reduce adequately and appropriately the risk of transmission of communicable disease.

Currently, all Whole Blood and blood components intended for transfusion and all Source Leukocytes intended for further manufacture are routinely tested for HBsAg and anti-HBc in order to reduce the risk of transmission of HBV (Refs. 1, 2, 3 and 5). In addition, all Source Plasma collections intended for further manufacture into plasma derivatives are routinely tested for HBsAg in order to reduce the risk of transmission of HBV in manufacturing pools of plasma derivatives.[2]

In the preamble to the final rule entitled "Requirements for Testing Human Blood Donors for Evidence of Infection Due to Communicable Disease Agents," published in the *Federal Register* of June 11, 2001 (66 FR 31146), we discussed the approved donor screening tests that we considered, as of that date, to be necessary to reduce adequately and appropriately the risk of transmission of HBV. We also stated that as technology advances, we intend to issue guidance describing those tests that we consider to reduce adequately and appropriately the risk of transmission of communicable disease agents. Accordingly, in this draft guidance document, we are notifying you that we consider FDA-licensed HBV NAT to be necessary to reduce adequately and appropriately the risk of transmission of HBV.

We note that the tests referenced in this document have been licensed by FDA for the screening of blood donors for HBV DNA and have the ability to detect the evidence of infection at an earlier stage than is possible using previously approved HBsAg and antibody to hepatitis B core antigen (anti-HBc) tests. Because FDA-licensed HBV NAT are now widely available, we recommend that establishments use these tests, in accordance with § 610.40.

A. Rationale for Donor Screening Using HBV NAT

Hepatitis B virus is a major human pathogen that may cause acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (Ref. 6). Most primary infections in adults are self-limited, the virus is cleared from blood and liver, and individuals develop a lasting immunity. Fewer than 5% of infected adults develop persistent asymptomatic infections (i.e., a carrier state). However, infants and young children have a much higher likelihood of developing a chronic hepatitis B infection than do older children and adults. According to data obtained in 2004 from the Centers for Disease Control and Prevention, about 1% of adults without other preexisting conditions are estimated to get chronic hepatitis B if infected, but 2% to 10% of children more than 5 years of age get chronic

[2] See Footnote 1.

hepatitis B, and 30% to 90% of children less than 5 years of age develop chronic hepatitis B if infected (Ref. 7). In addition, many patients receiving blood are immunocompromised because of their underlying disease and/or because of medications that suppress the immune system making them more susceptible to severe HBV infection than otherwise healthy individuals. About 20% of chronically infected individuals can develop cirrhosis. Chronically infected subjects have 100 times higher risk of developing hepatocellular carcinoma than non-carriers (Ref. 6).

Currently, HBV is transmitted by blood transfusions more frequently than hepatitis C virus (HCV) or human immunodeficiency virus (HIV). The residual risk of post-transfusion HBV infection is estimated to be about 1:357,000 to 1:280,000 per transfusion. In comparison, those risks for HIV and HCV are estimated to be 1:1,467,000 and 1:1,149,000, respectively (Ref. 8). Depending on the sensitivity of the test, implementation of HBV NAT has the potential to reduce risk to levels similar to those for HIV and HCV. HBV can be transmitted by blood from asymptomatic donors with acute HBV infections who have not yet developed HBsAg or anti-HBc (i.e., donors in the seronegative window period), when HBV DNA can be detected in the donor's blood (Refs. 9 and 10). Depending on the relative sensitivities of HBsAg and HBV NAT assays used, HBV DNA can be detected 2 to 5 weeks after infection, and up to 40 days (mean 6 to 15 days) before HBsAg (Ref. 7). HBV DNA levels rise slowly and are present at relatively low levels during the seronegative window period of early infection. HBV DNA can also be detected along with HBsAg and anti-HBc in chronic hepatitis B infections, and sometimes in recovered infections that are negative for HBsAg and positive for antibodies to hepatitis B surface antigen (anti-HBs) and anti-HBc (Refs. 6 and 11). Rarely, HBV DNA can be detected in the absence of HBsAg, anti-HBc and anti-HBs (Ref. 12).

Blood for transfusion in the United States (U.S.) is also tested for anti-HBc. Anti-HBc develops a few days after the appearance of HBsAg and usually remains detectable for life, irrespective of whether the individual recovers from acute hepatitis B or whether chronic HBV infection develops. Because of the availability and use of tests to detect anti-HBc, HBV NAT's potential utility in further reducing risk of hepatitis B transmission by blood transfusion is mainly restricted to the early HBsAg-negative phase of infection (i.e., a potential reduction of the infectious window period of up to 40 days depending on sensitivity of the HBsAg test).

There are currently three FDA-licensed HBV NAT assays for screening Whole Blood and blood components available in the U.S. Following licensure of the first HBV NAT assay in April 2005 (the Roche COBAS AmpliScreen HBV NAT that uses pools of up to 24 donation samples), FDA did not recommend use of HBV NAT. At that time, FDA's position on the use of HBV NAT was based, in part, upon discussions by the Blood Products Advisory Committee (BPAC or Committee) at the meeting on July 23, 2004 (Ref. 13), and on a recommendation from the Department of Health and Human Services Secretary's Advisory Committee on Blood Safety and Availability (ACBSA) on August 27, 2004 (Ref. 14). In making its recommendations, the ACBSA considered a number of broad public health issues including cost-effectiveness, feasibility, and overall public

health benefit, in addition to scientific data on detection of HBV in donors. One of FDA's reasons for not recommending HBV NAT at that time was the sensitivity of HBV NAT in the available format, when compared to the available serologic testing, did not provide sufficient additional safety to the blood supply to warrant recommending its use. FDA's reasoning was based on information that most blood establishments would have to test pools of 24 samples (thus diluting the individual samples by 1:24), because it was not feasible for most blood establishments to test single samples from donations or even small pools of samples.

Since licensure of the first HBV NAT in 2005, the following changes have occurred:

1. FDA has licensed two additional HBV NAT assays with indications for blood donor screening: Procleix® ULTRIO® Assay (Gen-Probe, Inc., San Diego, California), which uses up to 16 donation samples in a pool and COBAS TaqScreen MPX Test (Roche Molecular Systems, Inc., Pleasanton, California), which uses up to 6 donation samples in a pool. These multiplex assay systems can simultaneously detect HIV, HCV and HBV in a single donation, thus improving the feasibility of routine NAT testing for HBV. FDA has also licensed the UltraQualTM HBV PCR Assay (National Genetics Institute, Los Angeles, California), which provides results of HBV NAT of Source Plasma samples, or of plasma samples from Source Plasma donors at the time of donation. The assay is an "in-house" test; no kit is sold. The assay uses up to 512 donation samples in a pool.)

2. With the recent advance in technology and increased automation enabling the performance of NAT with smaller pools of samples and individual samples, more sensitive HBV NAT testing of blood donations is now possible, resulting in an increase in the number of window period HBV DNA positive/HBsAg negative units that could be detected.

3. There is now more information available on the role of vaccination of donors and recipients against HBV infection that indicates that protection for the long term is not absolute (i.e., breakthrough infections can occur in previously vaccinated individuals who are exposed to the virus) (Refs. 10 and 15). Breakthrough infections are characterized by HBV NAT positivity, the presence of HBV-neutralizing anti-HBs (developed as a result of hepatitis B vaccination), low viral load and lack of symptoms. HBsAg and anti-HBc may not subsequently develop or their appearance may be delayed. The infectivity of units obtained from hepatitis B-vaccinated donors with breakthrough HBV infections is unknown at the present time.

As mentioned above, in breakthrough HBV infections, HBsAg and anti-HBc development may be delayed or might not occur. Development is more likely to be detected by HBV NAT, particularly in the early stages of infection. As younger cohorts

in the population, who have received hepatitis B vaccine in a greater proportion than older cohorts (Refs. 16, 17, and 18), become eligible to donate blood, the proportion of vaccinated donors compared to non-vaccinated donors is expected to increase. Therefore, the proportion of donors with HBV breakthrough infections, compared to those with non-breakthrough, wild-type, HBV infections, would also be expected to increase. These donors' asymptomatic breakthrough infections are more likely to be detected by HBV NAT than to be detected by HBsAg or anti-HBc assays because HBsAg and anti-HBc development might be delayed or might not occur, even though HBV DNA is present and detectable by HBV NAT in the initial stage of the infection. In addition, HBV mutants appear to be more likely to be detected by HBV NAT than by HBsAg assays (Ref. 10).

Much of the available literature seem to indicate that HBV NAT positive/anti-HBs positive/HBsAg negative blood, irrespective of anti-HBc test results, does not transmit HBV (Refs. 11, 12, 19, 20, 21, and 22). However, there are at least two reports of such possible transmissions (Refs. 23 and 24), and one report that appears to have confirmed transmission of HBV by HBV NAT positive/anti-HBs positive/HBsAg negative blood (Ref. 25). Therefore, there can be no assumption of non-infectivity of units from donors with breakthrough infections containing HBV DNA and vaccine-induced, HBV-neutralizing anti-HBs when transfused into recipients. Nor can we assume a lack of morbidity and mortality in recipients, especially when many recipients are immunocompromised, as previously mentioned.

At the April 1, 2009 BPAC meeting (Ref. 26), the Committee agreed with FDA's position that there is no assumption of non-infectivity to recipients of units from donors with breakthrough infections. Therefore, in this guidance, we are recommending that all units of blood used for transfusion should be tested by an FDA-licensed HBV NAT. The Committee also supported FDA setting a sensitivity standard of 200 IU/mL HBV DNA for detection of HBV DNA in an individual donation when HBV NAT assays are used to test blood and blood components intended for transfusion. However, because of technological advances that have occurred since the time of the BPAC meeting in 2009, we are recommending a sensitivity standard of 100 IU/mL for HBV DNA detection in an individual donation (see section IV.A). Due to advances in technology and automation, FDA considers a sensitivity standard of 100 IU/mL to be attainable and practical for blood establishments that collect donations of Whole Blood and blood components intended for transfusion.

With regard to testing Source Plasma units for further manufacture into injectable plasma derivatives for HBV DNA, we believe that such testing adds another layer of safety for plasma derivatives by limiting the viral load in plasma pools for fractionation, in addition to viral inactivation and/or removal steps during their manufacture and the presence of neutralizing anti-HBs in manufacturing pools. During the BPAC meeting held on April 28, 2011 (Ref. 27), the Committee agreed with FDA that the available scientific data supports the concept that testing Source Plasma donations by HBV NAT increases the safety margin of plasma derivatives. Therefore, FDA is recommending that all units of Source Plasma intended for manufacture into injectable plasma derivatives be tested by

an FDA-licensed HBV NAT. In consideration of viral inactivation and removal in plasma fractionation, FDA is recommending a sensitivity standard of 500 IU/mL for detection of HBV DNA in an individual collection, rather than 100 IU/mL (see section IV.A). This sensitivity standard was endorsed by BPAC at the April 28, 2011 meeting (Ref. 27).

Similar to plasma derivatives, the HBV safety of products made from Source Leukocytes depends in large measure on viral removal and inactivation during manufacturing. However, since Source Leukocytes are obtained from Whole Blood donors, for consistency, we are also recommending a sensitivity standard of 100 IU/mL for HBV DNA detection in the individual donation of Source Leukocytes.

B. Donor Requalification

Under § 610.41(b), "[a] deferred donor subsequently may be found to be suitable as a donor of blood or blood components by a requalification method or process found acceptable for such purposes by FDA."[3]

At the July 21, 2005 BPAC meeting (Ref. 28), the Committee agreed with FDA's proposed requalification criteria for donors of Whole Blood and blood components for transfusion and Source Plasma for further manufacture, who tested reactive by HBV NAT, when a follow-up sample is tested using HBV NAT and serologic tests. Data presented at the meeting demonstrated that a 6-month follow-up period encompasses the pre-seroconversion window period with sufficient confidence that negative test results for HBsAg, anti-HBc and HBV DNA by NAT, after a 6-month period, rule out HBV infection. For purposes of reentry, we recommend that you use an FDA-licensed HBV NAT labeled as having a sensitivity of $\leq$2 IU/mL at 95% detection rate [1 IU = ~5 copies of HBV DNA/mL].[4] Donors with negative results for HBV DNA at this level of sensitivity are highly unlikely to be infected with HBV (Ref. 29). Depending upon the assay and the platform used, this sensitivity may only be achieved when testing individual donor samples. Recommended criteria for donor requalification are presented in section IV.C.

IV. RECOMMENDATIONS

A. Donor Screening Using HBV NAT

Under § 610.40(b), you must use screening tests that FDA has approved for such use, in accordance with the manufacturers' instructions. You must perform one or more such

[3] A deferred donor may serve as an autologous donor in accordance with § 610.40 and § 610.41. Note that a deferred donor who donates for autologous use is not deemed to be reentered and remains deferred, until the criteria for reentry are met.

[4] COBAS AmpliScreen HBV Test (Roche Molecular Systems, Inc., Pleasanton, California): Triplicate testing using the multiprep specimen processing procedure. See package insert.
Procleix® ULTRIO® Assay (Gen-Probe, Inc., San Diego, California): Testing 6 replicates. See package inserts.

tests as necessary to reduce adequately and appropriately the risk of transmission of communicable disease, including HBV.

1. In order to meet the requirement under § 610.40(b) for Whole Blood and blood components intended for transfusion and Source Leukocytes intended for further manufacture, we recommend that you use an FDA-licensed donor screening test for HBV DNA by NAT in addition to the detection of HBsAg and anti-HBc. If the FDA-licensed tests for detection of both HBsAg and anti-HBc are negative or non-reactive, we recommend that you test the donation further using an FDA-licensed HBV NAT that has a lower limit of detection of <100 IU/mL HBV DNA for HBV DNA detection in an individual donation. The FDA-licensed screening HBV NAT that you use may be in a minipool donation-sample testing format or an individual donation testing format, and may include multiplex NAT with testing of other agents, such as HIV and HCV or may be single virus NAT for HBV only. Testing for HBsAg, anti-HBc and HBV DNA by NAT may be performed concurrently.

2. In order to meet the requirement under § 610.40(b) for testing Source Plasma intended for further manufacture into plasma derivatives, we recommend that you use an FDA-licensed donor screening test for the detection of HBsAg. If the FDA-licensed test for detection of HBsAg is negative or non-reactive, we recommend that you test the donation further using an FDA-licensed HBV NAT that has a lower limit of detection of <500 IU/mL HBV DNA for HBV DNA detection in an individual donation. The FDA-licensed screening HBV NAT that you use may be in a minipool donation-sample testing format or an individual donation testing format, and may include multiplex NAT with testing of other agents, such as HIV and HCV, or may be single virus NAT for HBV only. Testing for HBsAg and HBV DNA by NAT may be performed concurrently. (FDA does not currently recommend that Source Plasma donors be tested for anti-HBc (Ref. 2)).

   As a general matter, under § 610.40(h)(1), if any of the FDA-licensed tests for the detection of either HBsAg or anti-HBc is reactive, the donation must be not be shipped or used.[5] In this instance, we believe that you have met the standard for adequate and appropriate screening for HBV and you do not need to test the unit further using an FDA-licensed HBV NAT. However, you may choose to test such a reactive donation by using an FDA-licensed HBV NAT to provide useful information to the donor, or if you wish to reenter the donor as described below in this Guidance.

   We note that in regard to HBsAg reactivity, as required by § 610.40(e), you must proceed to supplemental testing for HBsAg to determine

[5] Blood components that are reactive for HBsAg and/or anti-HBc may be shipped or used if they meet the conditions for an exception described in § 610.40(h)(2).

whether or not a reactive HBsAg test result can be confirmed positive, and is not a false positive (i.e., test result recorded HBsAg negative), using either an additional, more specific test, such as an HBsAg neutralization test or an HBV NAT assay with a limited supplemental test indication. Some HBV NAT assays have received this limited supplemental indication for repeatedly reactive HBsAg test results. If a donation tests HBV NAT-positive for HBV DNA using an HBV NAT with a limited supplemental test indication, and if that donation also tests HBsAg repeatedly reactive in a screening test, the HBsAg test result can be recorded as HBsAg positive. In this case, an HBsAg neutralization test need not be performed. However, if a donation tests HBV NAT-negative for HBV DNA using an HBV NAT with a limited supplemental test indication, and if that donation tests HBsAg repeatedly reactive in a screening test, an HBsAg neutralization test should be performed. In this case, the result of the neutralization test serves as the test of record (Ref. 1). We further note that there is no licensed supplemental, more specific, test for anti-HBc at the present time. Donors with anti-HBc reactive results may be requalified as described in Ref 3.

B. Management of Donors and Units Based on Hepatitis B Test Results

1. Donor and Unit Management When the HBV DNA NAT Result is Negative

   a. If a unit tests negative by individual donation NAT (ID-NAT) for HBV DNA or is part of a minipool that tests negative, then the donor and the unit should be managed consistent with FDA guidances and recommendations, as appropriate (Refs. 1 through 5), provided that the donor satisfies all applicable donor eligibility criteria and the unit is otherwise suitable for release.

   b. Units of Whole Blood and blood components may be used for transfusion and Source Leukocytes may be used for further manufacture that test negative for HBV using FDA-licensed HBV NAT, HBsAg, and anti-HBc assays, provided that the donor satisfies the donor eligibility criteria in § 640.3 (21 CFR 640.3), and that all other donor screening tests for communicable disease agents required in § 610.40(a) and (i) for Whole Blood and blood components, including Source Leukocytes, are negative, and the units are otherwise suitable for release.

   c. Units of Source Plasma and recovered plasma that test negative for HBV using FDA-licensed HBV NAT and HBsAg assays may be used for further manufacture, provided that the donor satisfies the donor eligibility criteria in § 640.63 (for Source Plasma) and § 640.3 (for recovered plasma), and that the requirements in § 610.40 are met and

all other screening tests for communicable disease agents required in § 610.40(a) and (i) are negative and the units are otherwise suitable for release (see footnote 1).

2. Donor and Unit Management when the HBV DNA NAT Result is Positive

   a. In accordance with § 610.40(h), except for autologous donations under § 610.40(h)(2)(i) or where you have obtained FDA's written approval for the shipment or use in accordance with § 610.40(h)(2)(ii)(A), you must not ship or use a unit of Whole Blood or blood components for transfusion, or a unit of Source Leukocytes for further manufacture that tests positive by HBV ID-NAT (either from direct screening by ID-NAT or from deconstruction of a NAT-positive minipool) (Table 1, Categories 1 through 6).

   b. In accordance with § 610.41, you must defer a donor who tests reactive for HBV, and in accordance with 21 CFR Part 630 (Part 630) you must notify the blood donor. You should permanently defer a donor of Whole Blood or blood components, or Source Leukocytes, whose NAT and serologic test results are as follows. (The donor is not eligible for reentry):

      i. HBV NAT-positive, HBsAg RR and confirmed positive, either by neutralization or when using a NAT with a limited supplemental claim, regardless of anti-HBc results (Table 1, Categories 1 and 2); or

      ii. HBV NAT-positive when using a NAT that does not have a limited supplemental claim and HBsAg RR is not confirmed by neutralization, and anti-HBc is RR (Table 1, Category 3).

   c. In accordance with § 610.41, you must defer a donor who tests reactive for tests for HBV, and in accordance with Part 630 you must notify the blood donor. You should indefinitely defer a donor of Whole Blood or blood components, including Source Leukocytes, whose NAT and serologic test results are as follows (The donor may be eligible for reentry, as described in section IV.C.):

      i. HBV NAT-positive, HBsAg non-reactive (NR), anti-HBc RR (Table 1, Category 4); or

      ii. HBV NAT-positive, and both HBsAg and anti-HBc are non-reactive (Table 1, Category 5); or

iii. HBV NAT-positive using a NAT that does not have a limited supplemental claim and HBsAg RR is not confirmed by neutralization, and is anti-HBc NR (Table 1, Category 6).

Table 1. Donor and Unit Management (Whole Blood and Blood Components for transfusion, and Source Leukocytes for Further Manufacture) when the HBV DNA NAT Result is Positive

Category	HBV NAT Result†	HBsAg Result	Anti-HBc Result	Donor and Unit
1	Positive	Repeat Reactive / Confirmed Positive*	Non-Reactive	Discard unit; Permanently defer donor Donor not eligible for reentry
2	Positive	Repeat Reactive / Confirmed Positive*	Repeat Reactive	
3	Positive	Repeat Reactive / Not Confirmed	Repeat Reactive	
4	Positive	Non-Reactive	Repeat Reactive	Discard unit; Indefinitely defer donor; Donor may be eligible for reentry
5	Positive	Non-Reactive	Non-Reactive	
6	Positive	Repeat Reactive / Not Confirmed	Non-Reactive	

† Using a screening test, as described in section IV.A.1.
*Using either an HBsAg neutralization test or an HBV NAT with a limited supplemental test indication, as described in section III and section IV.A.3.

d. In accordance with § 610.40(h), except where you have obtained FDA's written approval for the shipment or use in accordance with § 610.40(h)(2)(ii)(A), you must discard and not use for further manufacture a unit of Source Plasma that tests positive by HBV ID-NAT (Table 2, Categories 1 through 3).

e. In accordance with § 610.41, you must defer a donor who tests reactive for tests for HBV, and in accordance with Part 630 you must notify the blood donor. You should permanently defer a donor of Source Plasma whose donation tests HBV NAT-positive and is HBsAg RR, confirmed positive either by neutralization, or when using a NAT with a limited supplemental claim. The donor is not eligible for reentry (Table 2, Category 1).

f. In accordance with § 610.41, you must defer a donor who tests reactive for tests for HBV, and in accordance with 21 CFR 630 you must notify the blood donor. You should indefinitely defer a donor of Source Plasma whose donation tests HBV NAT-positive when using a NAT that does not have a limited supplemental claim, and is either HBsAg NR or is HBsAg is RR not confirmed by neutralization (Table 2, Categories 2 and 3). The donor may be eligible for reentry, as described in section IV.C.

Table 2. Donor and Unit Management (Source Plasma for Further Manufacture) when the HBV DNA NAT Result is Positive

Category	HBV NAT Result†	HBsAg Result	Donor and Unit
1	Positive	Repeat Reactive / Confirmed Positive*	Discard unit; Permanently defer donor Donor not eligible for reentry
2	Positive	Non-Reactive	Discard unit; Indefinitely defer donor; Donor may be eligible for reentry
3	Positive	Repeat Reactive / Not Confirmed	

† Using a screening test, as described in section IV.A.2.
* Using either an HBsAg neutralization test or an HBV NAT with a limited supplemental test indication, as described in section III, and section IV.A.3.

C. Requalification Methods for Donors on the Basis of HBV NAT and HBV Serologic Test Results on the Follow-Up Sample

For purposes of reentry, we recommend that you use an FDA-licensed HBV NAT having a sensitivity of < 2 IU/mL at 95% detection rate.

1. Requalification of a Donor of Whole Blood or Blood Components for Transfusion and Source Leukocytes for Further Manufacture

   To reenter an indefinitely deferred donor of Whole Blood or blood components for transfusion, or Source Leukocytes for further manufacture, a new sample should be obtained from the donor at least 6 months after the collection of the sample that gave test results described in section IV. B.2.c. (no donation is made at this time). You should perform follow-up testing using HBV NAT (having a sensitivity of $\leq$ 2 IU/mL at 95% detection rate), HBsAg and anti-HBc FDA-licensed assays.

   a. If the new follow-up sample tests positive by HBV NAT, regardless of HBsAg and anti-HBc test results, we recommend that you permanently defer the donor (Table 3, Category 1).

   b. If the new follow-up sample tests negative by HBV NAT and NR by HBsAg and anti-HBc assays, the donor may be reentered (i.e., the donor is eligible to donate in the future), provided the donor meets all donor eligibility criteria in § 640.3 (Table 3, Category 2).

   c. If the new follow-up sample tests negative by HBV NAT and RR by HBsAg and/or RR by anti-HBc, we recommend that you evaluate the

donor further as described in the FDA guidance documents cited in Refs. 1, 2 and 3 (Table 3, Category 3).

NOTE: If you wish to perform follow-up testing on a donor of Whole Blood or blood components for transfusion or a donor of Source Leukocytes for further manufacture who is deferred because of HBV NAT test results, you may do so before the end of the 6-month waiting period for donor notification purposes or for medical reasons. Negative test results on follow-up for HBsAg, anti-HBc and HBV DNA by NAT (sensitivity at 95% detection rate of $\leq$ 2 IU/mL), may be useful in donor counseling. However, only negative results for all three tests (HBsAg, anti-HBc and HBV NAT), obtained at least 6 months after the collection of the sample that gave test results described in section IV.B.2.c, would qualify the donor for reentry. If you obtain a reactive HBV NAT, or repeatedly reactive anti-HBc, or repeatedly reactive HBsAg that is positive by neutralization during this 6-month waiting period, the donor would not be eligible for reentry, and we recommend that you defer the donor permanently.

A donor of Whole Blood or blood components for transfusion, or a donor of Source Leukocytes for further manufacture who has been requalified as described above in section IV.C.1., may on subsequent occasions be indefinitely deferred because of HBV NAT reactive results. You may reenter such a donor into the donor pool by again following all the procedures described in section IV.C.1.

2. Requalification of a Donor of Source Plasma for Further Manufacture

To reenter an indefinitely deferred donor of Source Plasma, you should obtain a follow-up sample from the donor (no donation is made at this time) at least 6 months after the collection of the sample that gave the test results described in section IV.B.2.f. You should perform follow-up testing using HBV NAT (having a sensitivity of $\leq$2 IU/mL at 95% detection rate) and HBsAg FDA-licensed assays.

a. If a new follow-up sample tests positive by HBV NAT, regardless of the HBsAg test result, you should permanently defer the donor (Table 3, Category 1).

b. If a new follow-up sample tests negative by HBV NAT and NR by HBsAg, the donor is eligible to donate in the future, provided the donor satisfies all donor eligibility criteria in § 640.63 (Table 3, Category 2).

c. If a new follow-up sample tests negative by HBV NAT and RR HBsAg, you should evaluate the donor further, as described in the FDA documents cited in Ref. 1 (Table 3, Category 3).

NOTE: If you wish to perform follow-up testing on a donor of Source Plasma who is deferred because of HBV NAT test results, you may do so before the end of the 6-month waiting period for donor notification purposes or for medical reasons. Negative test results on follow-up for HBsAg and HBV DNA by NAT (sensitivity at 95% detection rate of $\leq$ 2 IU/mL), may be useful in donor counseling. However, only negative results for both tests (HBsAg and HBV NAT), obtained at least 6 months after the collection of the sample that gave the test results described in section IV.B.2.f, would qualify the donor for reentry. If you obtain a reactive HBV NAT, or repeatedly reactive HBsAg that is positive by neutralization, the donor would not be eligible for reentry, and we recommend that you defer the donor permanently.

A donor of Source Plasma who has been requalified as described above in section IV.C.2., may on subsequent occasions be indefinitely deferred because of HBV NAT positive results. You may reenter such a donor into the donor pool by again following all procedures described in section IV.C.2.

Table 3. Reentry of Donors of Whole Blood and Blood Components for Transfusion or Further Manufacture on the Basis of HBV NAT and HBV Serologic Test Results on the Follow-Up Sample

For purposes of reentry, we recommend that you use an FDA-licensed HBV NAT labeled as having a sensitivity of $\leq$2 IU/mL at 95% detection rate.

Category	HBV NAT Result (sensitivity of $\leq$2 IU/mL at 95% detection rate)	HBsAg and/or Anti-HBc Result (Anti-HBc not required for SP)	Donor
1	Positive	Any test result	Permanently defer donor
2	Negative	Non-Reactive	Donor may be eligible for reentry
3	Negative	Repeat Reactive	For further evaluation, see FDA guidance documents that discuss donor testing for HBsAg and anti-HBc. Refs. 1, 2 and 3.)

3. Management of Donors and Units with Non-Discriminated Reactive Test Results

If you obtain a reactive Multiplex HIV-1 RNA/HCV RNA/HBV DNA NAT result on an individual donor sample (ID-NAT), and if the Discriminatory NATs are non-reactive for HIV-1 RNA, HCV RNA and HBV DNA, the sample is "Non-Discriminated Reactive." The unit must be quarantined and destroyed (§ 610.40(h)), or, if released for research or further manufacture, be appropriately relabeled as described in section IV.C. The donor must be deferred (§ 610.41). Note that the donor should be deferred for 6 months and is eligible for reentry after the 6-month waiting period. If you choose to reenter the donor, you may do so at the time of a donation without prior testing of a follow-up sample.

V. LABELING

A. Circular of Information for Whole Blood and Blood Components Intended for Transfusion

Consistent with other donor screening tests, the instruction circular, also known as the "Circular of Information", must be updated to state that an FDA-licensed NAT for HBV DNA was used to screen donors and that the results of testing were negative (§ 606.122(h)). We recommend that you use the following statement on the labeling for donations that test Non-Reactive:

> "Licensed nucleic acid test (NAT) for HBV DNA has been performed and found to be Non-Reactive."

B. Blood Components Intended for Further Manufacture

Upon implementation of an FDA-licensed NAT, we recommend that you use the following statement on the labeling for blood components intended for further manufacture into injectable or non-injectable products that test Non-Reactive:

> "Non-Reactive for HBV DNA."

See paragraph C of this section for recommendations for donations that test Reactive for HBV.

C. Reactive Units and Product Disposition

NAT reactive units must not be shipped or used, except as provided in § 610.40(h)(2). If released for these uses, the units must be relabeled consistent with the labeling requirements in §§ 606.121, 610.40 and 640.70. Thus, for example, you must label the reactive unit with the "BIOHAZARD" legend and with the following cautionary statements, as applicable:

> "Reactive for HBV DNA,"

and

> "Caution: For Further Manufacturing into In Vitro Diagnostic Reagents For Which There Are No Alternative Sources."

In addition, you should label the reactive unit with the following legend, if applicable:

> "Caution: For Laboratory Research Use Only."

VI. REPORTING CHANGES TO AN APPROVED APPLICATION

Under 21 CFR 601.12 (§ 601.12), FDA-licensed blood establishments are required to report changes to an approved biologics license application to FDA. FDA-licensed blood establishments must report the changes in paragraphs A, B, and C.1 and C.2.a of this section, as described below. However, except as specified in paragraph C.2.b of this section, unlicensed blood establishments are not required to report the changes to FDA.

A. Test Implementation

1. If you begin using an FDA-licensed NAT for the detection of HBV DNA in your facility according to the manufacturer's instructions, you must notify FDA of the testing change in your annual report (AR), in accordance with § 601.12(d), indicating the date that the revised standard operating procedures were implemented.

2. If you are already approved to use a registered contract donor testing laboratory to perform infectious disease testing of Whole Blood and blood components, including Source Plasma and Source Leukocytes, and the contract testing laboratory will now perform a NAT for HBV DNA, you must report this change in your AR (§ 601.12(d)).

3. If you will use a new contract testing laboratory to perform a NAT for HBV DNA, report as follows:

   a. If the new testing laboratory is registered with FDA and has been performing infectious disease testing for Whole Blood and blood components, including Source Plasma and Source Leukocytes, report this as a Changes Being Effected (CBE) Supplement, in accordance with § 601.12(c)(5).

   b. If the new testing laboratory has not previously performed infectious disease testing for blood products, you must report this as a Prior Approval Supplement (PAS), in accordance with § 601.12(b). The

new testing laboratory must register with FDA in accordance with 21 CFR Part 607 and § 610.40(f).

B. Labeling

Labeling refers to the instruction circular (e.g., Circular of Information) required under § 606.122 and the container labels on blood or blood components required under, among other provisions, §§ 606.121, 610.40 and 640.70.

1. If you revise your labeling to include the statements in this guidance in their entirety and without modification, you must report this change as a CBE labeling supplement in accordance with § 601.12(f)(2)).

2. If you revise your labeling to include alternative statements, you must report this change as a PAS labeling supplement in accordance with § 601.12(f)(1).

C. Procedures for Requalification of Donors

1. We consider the implementation of recommendations in this guidance in their entirety and without modification to be a minor change to an approved license application. Therefore, FDA-licensed establishments are not required to have FDA prior approval and may submit a statement of this change in their AR under § 601.12(d), indicating the date that the revised standard operating procedures were implemented.

2. Under § 610.41(b), you may only re-enter a previously deferred donor using a requalification method found acceptable by FDA for such purposes. We consider the requalification methods described in this guidance to be acceptable. If you choose to use an alternative requalification method, you must report this as follows:

   a. FDA-licensed blood establishments must submit the alternative requalification method as a PAS (§ 601.12(b)).

   b. Unlicensed blood establishments must submit the alternative requalification method to FDA before it is implemented so that we may determine whether it is acceptable.

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研究報告の概要

○日本における年齢及び地域別有病率によるC型肝炎、B型肝炎の診断未確定キャリア総数の推定
目的: 日本でC型肝炎ウイルス(HCV)、B型肝炎ウイルス(HBV)の診断未確定キャリアの総数を推定すること。
方法: 地域及び年齢別のHCVならびにHBV有病率は、20-39歳については初回供血者(n=2,429,364)、40-74歳については定期健康診断の受診者(HCV;n=6,204,968、HBV;n=6,228,967)にて調査された。5-19歳の若者の有病率は一つの県において決定され(HCV;n=79,256、HBV;n=68,792)、75歳以上の高齢者のHCV有病率は指数モデルにて推定された。HBV感染はHBs抗原の検出により決定し、HCV感染はアルゴリズムまたは抗HCV抗体を持つ個人の70%が持続性感染であると仮定する事のどちらかにより決定した。
結果: 2005年の127,285,653人の全人口のうち、807,903人(95%CI; 679,886-974,292)がHCVキャリアであると算出され(0.63%)、903,145人(95%CI; 837,189-969,572)がHBVキャリアであると算出された(0.71%)。C型肝炎の年齢による特徴は、年齢とともに有病率の変動幅が大きくなることである。地域別の特徴は、有病率の変動幅が大きく異なり、西日本で増幅が大きい傾向があることである。一方B型肝炎では、全地域において55~59歳で最も有病率が高く、北海道では3.1%に上った。どの地域においても2000年と2005年を比較すると、HCV及びHBVキャリアの割合は減少している。
結論: 一般集団におけるHCV及びHBVの診断未確定感染者の正確な評価は、肝疾患の発症の可能性を予測し、健康管理の改善に対する適切な対策に寄与する。

使用上の注意記載状況・その他参考事項等

新鮮凍結血漿「日赤」
新鮮凍結血漿-LR「日赤」
新鮮凍結血漿-LR「日赤」成分採血

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
日本におけるHCV、HBVキャリアの総数は、全人口のうちHCVは約81万人(0.63%)、HBVは約90万人(0.71%)であると算出されたとの報告である。	HBV、HCV感染に関する新たな知見等について、今後も情報の収集に努める。

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Intervirology

Total Numbers of Undiagnosed Carriers of Hepatitis C and B Viruses in Japan Estimated by Age- and Area-Specific Prevalence on the National Scale

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Key Words
Hepatitis C virus · Hepatitis B virus · Blood donors · Liver cirrhosis · Hepatocellular carcinoma · Healthcare · Japan

Abstract
Objective: To estimate total numbers of undiagnosed carriers of hepatitis C virus (HCV) and hepatitis B virus (HBV) in Japan. ***Methods:*** Area- and age-specific prevalence of HCV as well as HBV was determined in the first-time blood donors [20-39 years (n = 2,429,364)] and examinees of periodical health check-ups [40-74 years (6,204,968 for HCV and 6,228,967 for HBV)] in Japan. Prevalence in adolescents [5-19 years (79,256 for HCV and 68,792 for HBV)] was determined in a single prefecture, and that of HCV in the elderly (≥75 years) was estimated by the exponential model. HBV infection was determined by the detection of hepatitis B surface antigen, and HCV infection by either the algorithm or assuming persistent infection in 70% of the individuals with antibody to HCV. ***Results:*** Of the total population of 127,285,653 in 2005, 807,903 (95% CI 679,886-974,292) were estimated to be infected with HCV at a carrier rate of 0.63%, and 903,145 (837,189-969,572) with HBV at that of 0.71%. ***Conclusion:*** Accurate estimation of undiagnosed HCV and HBV carriers in the general population would help to predict the future burden of liver disease, and take appropriate measures for improving healthcare.

Introduction

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are estimated to infect 170 and 350 million people over the world, respectively [1, 2]. Most infections with HCV or HBV do not induce clinical liver disease, while ~30% of them develop severe liver disease such as cirrhosis and hepatocellular carcinoma [3, 4]. Hence, there is a pressing need to identify the individuals who have undiagnosed HCV or HBV infection, and take effective measures for terminating viral infections and preventing the progression of liver disease.

For management of persistent HCV and HBV infections in a given country, it is necessary to know their exact numbers for assessing medical and financial needs in the foreseeable future. Prevalence of undiagnosed HCV or HBV

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infection has been estimated by survey of blood donors in Japan [5] and a representative population in the USA [6].

In the present study, area- and age-specific prevalence of HCV or HBV infection was determined in 8 jurisdiction areas of the Japanese Red Cross Blood Center. Then, the total numbers of undiagnosed HBV and HCV infections were estimated by compiling the results in the first-time blood donors and examinees of the periodical health check-up program. Of the 127,285,653 Japanese registered in 2005, 807,903 (0.63%) were estimated to be infected with HCV and 903,145 (0.71%) with HBV.

Materials and Methods

Japanese Population

Japan is divided into 8 areas, along its north-to-south axis, according to jurisdiction of the Japanese Red Cross Blood Center, into Hokkaido, Tohoku, Kanto, Hokuriku/Tokai, Kinki, Chug Shikoku and Kyushu. Populations in 5-year age groups in (jurisdiction area were obtained from the registry at the National Census 2005.

First-Time Blood Donors

During 6 years from January 2001 to December 2006, 3,748,422 individuals (aged 16–64 years) donated whole blood or apheresis products for the first time, and their sera were tested for markers of HCV and HBV infections. Ongoing HCV infection was estimated by assuming the detection of HCV RNA in 70% of individuals with the antibody to HCV (anti-HCV), in accordance with a previous report [5].

Examinees of Hepatitis Virus Infections

Since the fiscal year 2002 in Japan, individuals who turned 40, 45, 50, 55, 60, 65 and 70 years were offered to take tests for hepatitis viruses at periodical health check-ups by a 5-year national project. During 5 years through 2006, 6,204,968 individuals received tests for HCV and 6,228,967 for HBV, corresponding to ~30% of the eligible Japanese, and their area- and age-specific prevalence of HCV or HBV infection was determined.

School Children and Adolescents

In the Iwate prefecture located in the north of Japan, biochemical markers of diseases dependent on the lifestyle were examined in children and adolescents at the entrance to schools. Their serum samples had been stored frozen, and were tested for markers of hepatitis virus infections. Carrier rates of HCV and HBV among them were calculated, with their ages adjusted to those in 2005; infants aged <5 were represented by the children aged from 5 to 9 years. Designs and procedures of this investigation were approved by the Ethics Committee of Hiroshima University.

Simulation of HCV and HBV Infections in the Elderly

By its age-specific profile, the prevalence of HCV was deduced to be an exponential function of the age. Accordingly, age-specific prevalence of HCV in the individuals aged ≥75 years was simulated by an exponential function model; it was constructed on the prevalence of HCV in each age group ≥50 years.

The formula was constructed as:

$$\log y(x) = a + bx$$

where x is the 5-year age code, $y(x)$ is an estimator of HCV prevalence in x, and a and b are coefficients.

The equation is transformed into:

$$y(x) = e^a e^{bx}$$

in which e^a represents the HCV prevalence when $x = 0$ (in the group aged 0–4 years), since $y(0)$ is equal to e^a. By replacing x for $x + 1$ in the above equation, it is converted to $y(x + 1) = e^a e^{b(x+1)}$.

Then, the following equation can be constructed:

$$y(x + 1) = e^b y(x)$$

where e^b is the slope of HCV prevalence increasing with age. Thus, the HCV prevalence is multiplied by a factor e^b for an increment of the age code by 1.

The simulation model was applied to estimate age-specific prevalence of HCV in each of 8 areas in the individuals ≥75 years.

Prevalence of HBV in the individuals ≥75 years was represented by that in those aged 70–74 years, since it stayed constant from 65 through 75 years.

Markers of Hepatitis Virus Infections

In blood donors, anti-HCV was determined by passive hemagglutination of the second generation with commercial assay kits (HCV PHA; Abbott Laboratories, North Chicago, Ill., USA) with a cutoff limit set at 2^5, as well as by particle agglutination with commercial assay kits (HCV PA Test-II; Fujirebio, Inc., Tokyo, Japan). HBsAg was determined by reversed passive hemagglutination with reagents prepared by the Japanese Red Cross.

In examinees of periodical health check-ups, ongoing HCV infection was determined by the algorithm with anti-HCV and HCV RNA [7]. Anti-HCV was determined by passive hemagglutination of the second generation with commercial assay kits (HCV PHA; Abbott Laboratories), and since 2002, it was determined by enzyme immunoassay with commercial assay kits (AxSYM HCV Dinapack-III; Abbott Laboratories). Samples with high anti-HCV titers contain HCV RNA, and therefore, only those with low and middle titers were examined for HCV RNA. HBsAg was determined by reversed-passive hemagglutination with commercial assay kits (Institute of Immunology Co., Ltd, Tokyo, Japan).

Statistical Analyses

Statistical analyses for the evaluation of R^2 values were performed with JMP 8.0 (SAS Institute, Inc., Cary, N.C., USA) and DeltaGraph 5.5 (RedRock Software, Inc., Salt Lake City, Utah, USA). A p value > 0.05 was considered significant.

Results

Age-Specific Prevalence of HCV in the First-Time Blood Donors and Examinees of Periodical Health Check-Ups

Figure 1 illustrates age-specific prevalence of HCV in the first-time blood donors (aged 15–69 years in 2005) and examinees of periodical health check-ups (39–73 years in 2005); 70% of individuals with anti-HCV were considered to possess HCV RNA in serum [5]. Results of two distinct populations were well in accord. For the first-time blood donors, however, the variation (95% CI) widened increasingly with age. It would have reflected decreases in the first-time blood donors with age, since the majority of these (83.5%) were aged ≤39 years. As the prevalence of HCV in blood donors ≥40 years was unreliable in them, that in examinees of periodical check-ups was adopted for estimating the national prevalence of HCV.

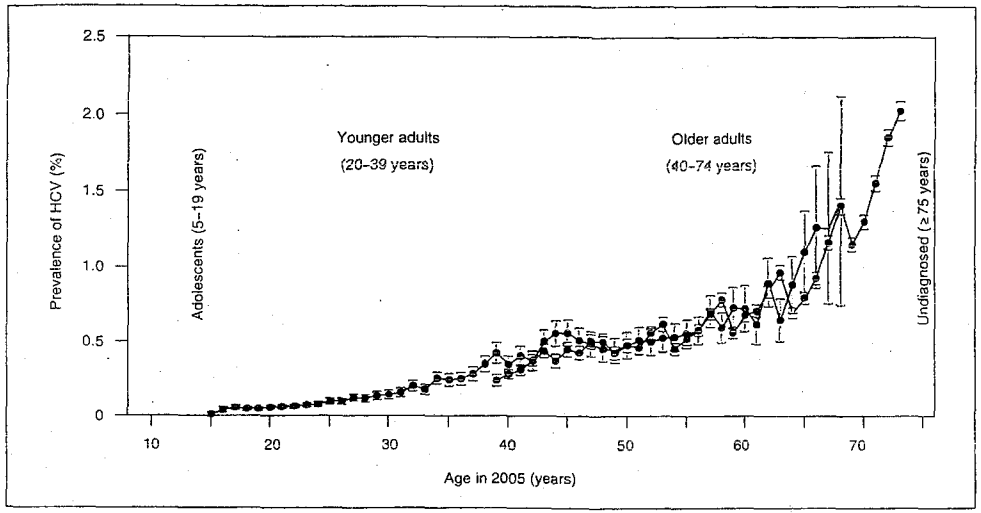

Fig. 1. Age-specific prevalence of HCV in Japan. The prevalence of HCV was determined in the first-time blood donors aged from 15 to 68 years (blue dots) and examinees of periodical health check-ups aged from 39 to 73 years (red dots). Their ages were adjusted to those in the year 2005. Bars indicate ranges of 95% CI.

Area-Specific Prevalence of HCV in Eight Jurisdiction Areas

In view of distinct geographic distribution of HCV, the prevalence of HCV in the general population would not be applicable to every area in Japan. Figure 2 compares results in the first-time blood donors and recipients of health check-ups among 8 jurisdiction areas spanning from north (Hokkaido) to south (Kyushu). They unfolded a wide variety in the age-specific prevalence of HCV. Although the prevalence of HCV increased with age in all areas, the slope of increase differed widely among them. Hence, it was necessary to employ a distinct age-specific prevalence in each of the 8 areas for estimating HCV carriers precisely.

Table 1. Age-specific prevalence of HCV in three different populations

Age in 2005	n	HCV-positive, n	Prevalence, % (95% CI)
School children			
5–9	17,390	2	0.012 (0.000–0.027)
10–14	29,817	3	0.010 (0.000–0.021)
15–19	32,049	7	0.022 (0.006–0.038)
Blood donors			
20–24	1,205,966	1,122	0.065 (0.061–0.070)[a]
25–29	536,560	874	0.114 (0.105–0.123)[a]
30–34	408,814	1,089	0.186 (0.173–0.200)[a]
35–39	278,024	1,190	0.300 (0.279–0.320)[a]
HCV screening			
40–44	611,146	2,127	0.348 (0.333–0.363)
45–49	495,032	2,292	0.463 (0.444–0.482)
50–54	675,350	3,485	0.516 (0.499–0.533)
55–59	947,438	5,974	0.631 (0.615–0.646)
60–64	1,081,854	8,423	0.779 (0.762–0.795)
65–69	1,264,496	13,722	1.085 (1.067–1.103)
70–74	1,054,472	17,649	1.674 (1.649–1.698)

[a] The prevalence in blood donors was based on an assumption of HCV infection persisting in 70% of those with anti-HCV [5].

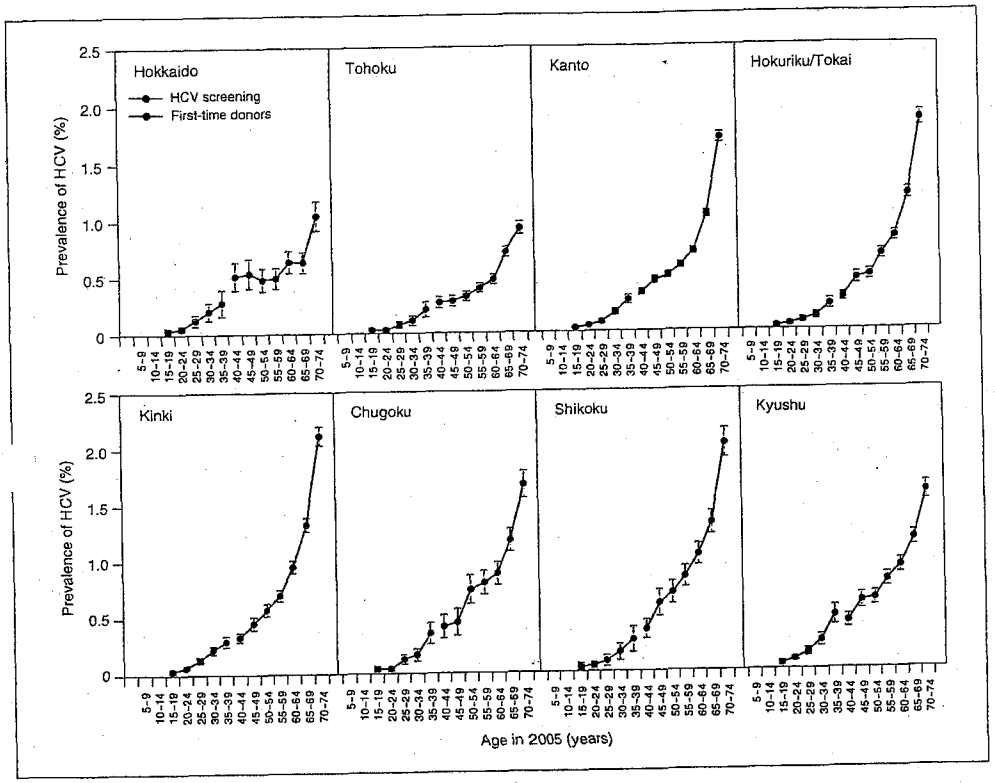

Fig. 2. Age-specific prevalence of HCV in 8 jurisdiction areas in Japan. The prevalence of HCV is calculated in each of twelve age groups notched by 5 years. The prevalence in five groups ≤39 years was represented by the first-time blood donors, and that in seven groups ≥40 years by recipients of HCV screening. Bars indicate ranges of 95% CI.

Prevalence of HCV in Adolescents

Since blood donors were restricted to 16–64 years of age, and health examinees were targeted on 40–70 years, they did not cover individuals aged ≤15 or ≥75 years in the year 2005. To fill in an opening on the younger side, the age-specific prevalence of HCV was determined in school children and adolescents in the Iwate prefecture (table 1). The prevalence in infants aged 0–4 years was assumed similar to that in the children aged 5–9 years; an extremely low prevalence of HCV (0.012%) would support such an assumption.

Simulating Prevalence of HCV in the Elderly

The prevalence of HCV appeared to be an exponential function of the age, according to its profiles in the first-time blood donors and examinees of health check-ups (fig. 1). Based on this assumption, a formula was constructed to simulate the prevalence of HCV in age groups ≥75 years for each of the 8 jurisdiction areas in Japan (see Materials and Methods).

Figure 3 compares actual (dots) and simulated data (red line) of five age groups from 50 to 74 years (corresponding to age codes 10–14) among the 8 areas. There was a high coefficient of determination between them,

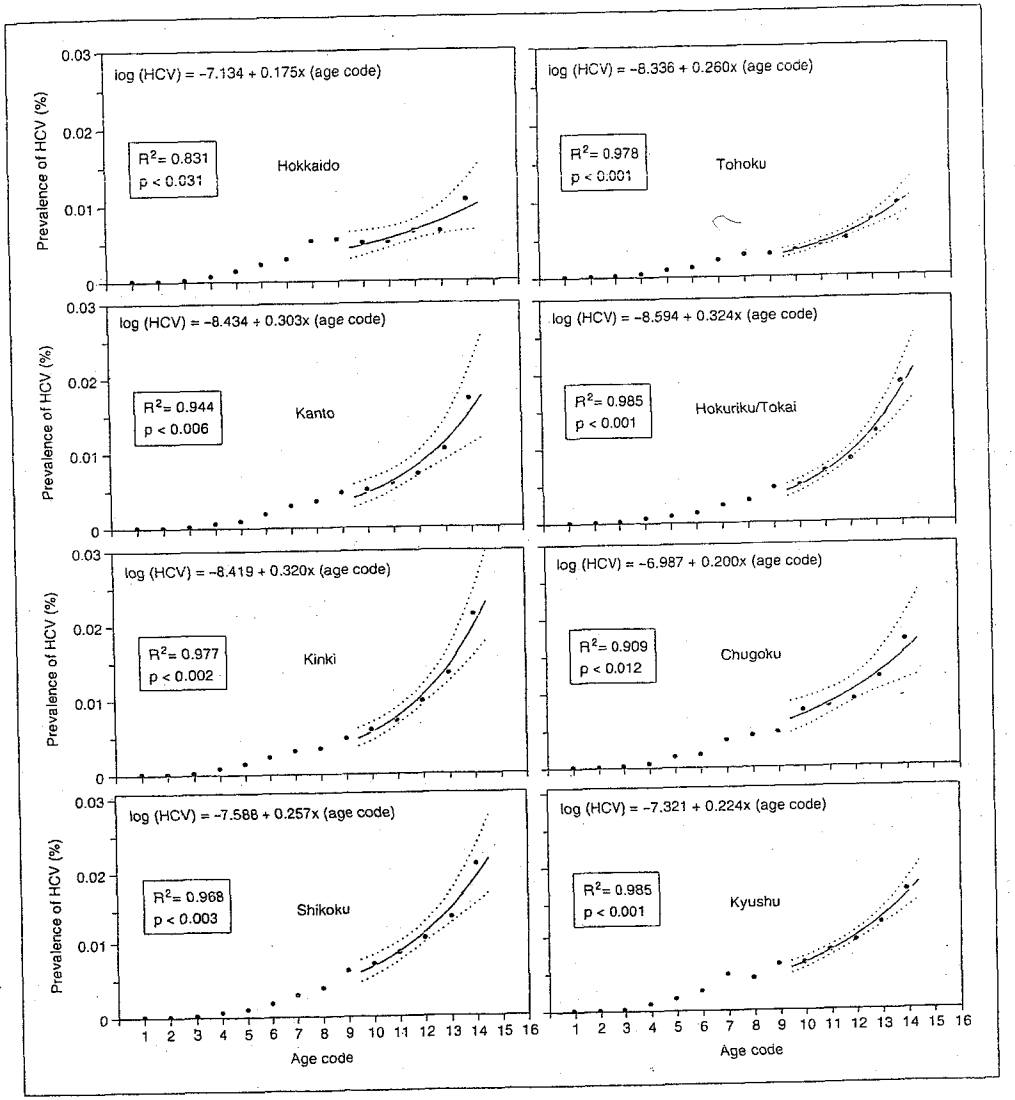

Fig. 3. Simulation of age-specific prevalence of HCV in the elderly. Prevalence of HCV in the first-time blood donors as well as examinees of periodical health check-ups (dots) and that simulated by formulation (red line with ranges of 95% CI in dotted line) are shown for 8 jurisdiction areas in Japan. Formula is shown at the top of each area. Age codes are: 1, 5–9 years; 2, 10–14 years; 3, 15–19 years; 4, 20–24 years; 5, 25–29 years; 6, 30–34 years; 7, 35–39 years; 8, 40–44 years; 9, 45–49 years; 10, 50–54 years; 11, 55–59 years; 12, 60–64 years; 13, 65–69 years; 14, 70–74 years, and 15, 75–79 years.

Table 2. Regional and total HCV carriers in Japan

Areas	Population	HCV carriers (95% CI)	Carrier rate
Hokkaido	5,620,813	26,097 (19,356–34,413)	0.46%
Tohoku	12,047,975	50,688 (42,754–59,953)	0.40%
Kanto	41,247,892	235,328 (195,408–293,611)	0.57%
Hokuriku/Tokai	19,294,443	132,434 (114,216–154,446)	0.69%
Kinki	22,657,542	173,808 (147,548–207,173)	0.52%
Chugoku	7,650,977	53,296 (42,299–67,698)	0.70%
Shikoku	4,083,698	35,159 (28,746–43,004)	0.86%
Kyushu	14,682,313	101,092 (89,379–113,993)	0.80%
Total	127,285,653	807,903 (679,886–974,292)	0.63%

Table 3. Age-specific prevalence of HBV in three different populations

Age in 2005	n	HBV-positive, n	Prevalence, % (95% CI)
School children			
5–9	17,363	3	0.017 (0.000–0.037)
10–14	29,817	14	0.047 (0.022–0.072)
15–19	32,049	12	0.037 (0.016–0.059)
Blood donors			
20–24	1,205,966	1,826	0.151 (0.144–0.158)
25–29	536,560	1,650	0.308 (0.293–0.322)
30–34	408,814	1,759	0.430 (0.410–0.450)
35–39	278,024	1,327	0.477 (0.452–0.503)
HBV screening			
40–44	613,960	5,491	0.894 (0.871–0.918)
45–49	497,589	5,373	1.080 (1.051–1.109)
50–54	679,893	8,700	1.280 (1.253–1.306)
55–59	950,508	12,891	1.356 (1.333–1.379)
60–64	1,085,119	13,282	1.224 (1.203–1.245)
65–69	1,268,304	12,406	0.978 (0.961–0.995)
70–74	1,057,469	9,545	0.903 (0.885–0.921)

with R^2 values ranging from 0.831 to 0.985 ($p < 0.031$ and $p < 0.001$, respectively), attesting to the validity of this simulation. Of note, the factor *b* in formula (by which age codes were multiplied) varied broadly among the 8 areas. Thus, it was the highest in Hokuriku/Tokai at 0.324 and lowest in Hokkaido at 0.175, with close to twofold differences between them.

Estimation of Undiagnosed HCV Carriers in Eight Areas and the Entire Nation

Based on age- and area-specific prevalence of HCV, numbers of undiagnosed HCV carriers were calculated for 8 jurisdiction areas, and they were compiled in the entire nation (table 2). The prevalence of HCV in each of three age groups (75–79, 80–84 and ≥85 years) was simulated by the formula, while that of HBV was represented by the prevalence in the group of 70–74 years. As of the year 2005, 127,285,653 were registered in the national census of Japan, and 807,903 of these are estimated to have undiagnosed HCV infection at an overall carrier rate of 0.63%. There was an increasing gradient in the prevalence of HCV along the north-to-south axis of Japan.

Age-Specific Prevalence of HBV

Figure 4 depicts age-specific prevalence of HBV in 2005. It was deduced from HBsAg in the first-time blood donors (15–69 years) and examinees of periodical health check-ups (39–73 years). Since the prevalence of HBV in the elderly did not increase with age so sharply as that of HCV (fig. 1), it was presumed not to increase further and stay around 1% in the individuals ≥75 years. The age-specific prevalence of HBV tabulated in three different

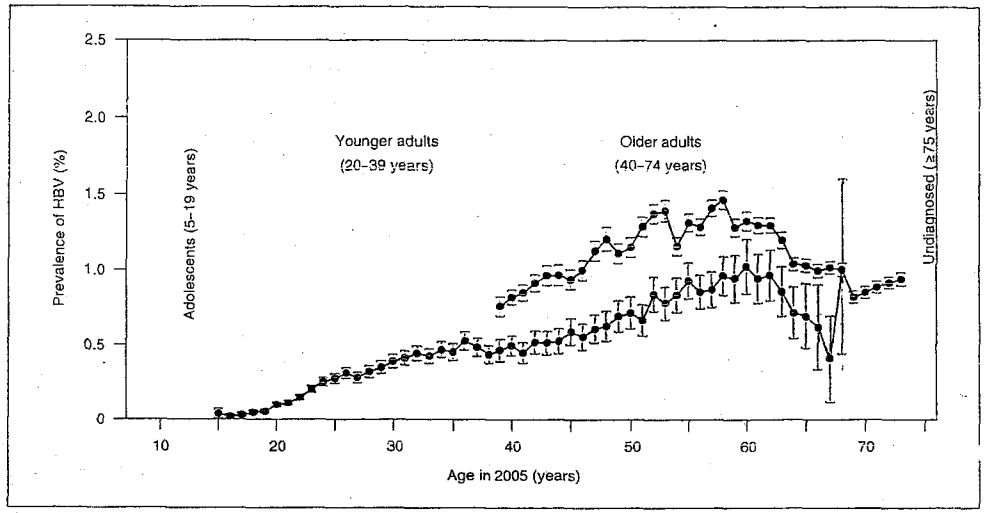

Fig. 4. Age-specific prevalence of HBV in Japan during 2002–2006. The prevalence of HBV was determined in the first-time blood donors aged from 15 to 68 years (blue dots) in the year 2005 and examinees of periodical health check-ups aged from 39 to 73 years (red dots) in the year 2005. Bars indicate ranges of 95% CI.

populations is listed in table 3. There was a constant decline with decreasing age in the frequency of HBV in individuals ≤39 years, and it was particularly low in children ≤9 years (0.017%).

In examinees of periodical health check-ups, the age-specific prevalence of HBV did not diverge and stayed within a narrow 95% CI (fig. 4). By contrast, that in the first-time blood donors dispersed widely. Such a variation in the age-specific prevalence of HBV would have been ascribed to the first-time blood donors who clustered in age groups ≤40 years.

Area-Specific Prevalence of HBV in Eight Jurisdiction Areas

The age-specific prevalence of HBsAg varied widely among 8 jurisdiction areas (fig. 5). HBsAg was most frequent in the age group of 55–59 years in every area, and reached 3.1% in the northern-most Hokkaido. The peak frequency decreased in central Japan (1.1% in Kanto and Hokuriku/Tokai), and increased towards the southern end (1.9% in Kyushu). Thus, the prevalence of HBsAg was determined individually along the axis of Japan in estimating the total number of HBV carriers in Japan.

Estimation of Undiagnosed HBV Carriers in Eight Areas and the Entire Nation

Numbers of undiagnosed HBV carriers were complied by multiplying age-specific prevalence of HBsAg by corresponding subpopulations in 8 jurisdiction areas (table 4). In total, 903,145 of the 127,285,653 (0.71%) individuals are estimated to have undiagnosed HBV infection in Japan in 2005.

Shift of Undiagnosed HCV and HBV Carriers during 5 Years (2000–2005) in Japan

Table 5 compares numbers of HCV and HBV carriers aged 15–69 years between 2000 and 2005 for 8 jurisdiction areas in Japan. Data for the year 2000 were extracted from a previous survey [5]. Data for the year 2005 were obtained in the first-time blood donors during 2001–2006 in this study by the same method as in the previous survey [5]. Undiagnosed HCV and HBV carriers decreased during 5 years by 55 and 47.5%, respectively. The overall carrier rate of HCV declined sharply from 0.95 to 0.44%, and that of HBV from 1.04 to 0.55% in Japan.

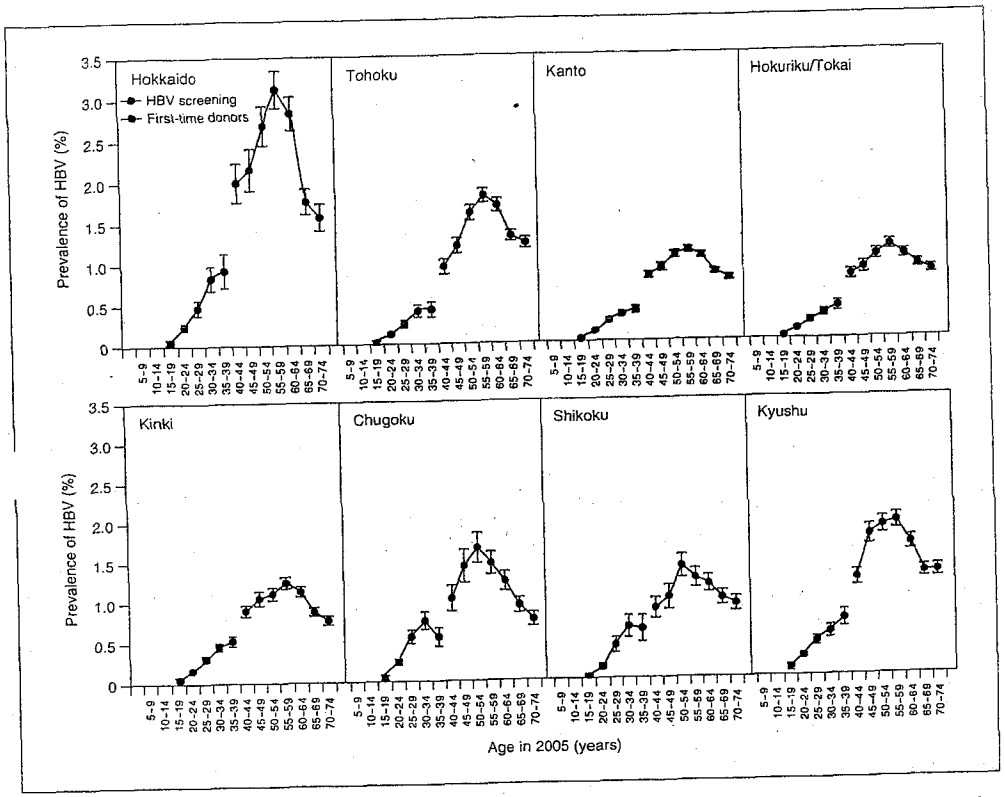

Fig. 5. Age-specific prevalence of HBV in 8 jurisdiction areas in Japan. The prevalence of HBV is calculated in each of twelve age groups notched by 5 years. The prevalence in five groups ≤39 years was represented by the first-time blood donors and that in seven groups ≥40 years by recipients of HCV screening. Bars indicate ranges of 95% CI.

Table 4. Regional and total HBV carriers in Japan

Areas	Population	HBV carriers (95% CI)	Carrier rate
Hokkaido	5,620,813	80,573 (72,314–88,765)	1.43%
Tohoku	12,047,975	104,736 (97,742–111,816)	0.87%
Kanto	41,247,892	231,799 (220,129–244,105)	0.56%
Hokuriku/Tokai	19,294,443	109,709 (101,722–117,581)	0.56%
Kinki	22,657,542	144,965 (134,387–155,464)	0.64%
Chugoku	7,650,977	59,948 (52,705–67,121)	0.78%
Shikoku	4,083,698	29,776 (26,080–33,437)	0.73%
Kyushu	14,682,313	141,639 (132,111–151,282)	0.96%
Total	127,285,653	903,145 (837,189–969,572)	0.71%

Table 5. Decrease of undiagnosed HCV and HBV carriers in the 15- to 69-year-old population in Japan

	Survey in 2000[a]		Survey in 2005		Difference	
	number estimated	carrier rate in area[b]	number estimated	carrier rate in area[b]	number estimated	balance
Shift of HCV carriers during 5 years from 2000 to 2005						
Hokkaido	41,139	0.99%	17,658	0.44%	−23,481	−57.1%
Tohoku	61,658	0.71%	30,525	0.37%	−31,133	−50.5%
Kanto	277,644	0.90%	126,283	0.41%	−151,361	−54.5%
Hokuriku/Tokai	88,724	0.64%	48,360	0.35%	−40,364	−45.5%
Kinki	178,871	1.06%	70,526	0.43%	−108,345	−60.6%
Chugoku	72,431	1.32%	24,595	0.47%	−47,836	−66.0%
Shikoku	43,497	1.49%	16,504	0.59%	−26,993	−62.1%
Kyushu	120,989	1.16%	64,115	0.63%	−56,874	−47.0%
Total	884,954	0.95%	398,567	0.44%	−486,387	−55.0%
Shift of HBV carriers during 5 years from 2000 to 2005						
Hokkaido	106,896	2.56%	54,557	1.35%	−52,339	−49.0%
Tohoku	104,923	1.21%	48,490	0.58%	−56,433	−53.8%
Kanto	255,207	0.83%	132,414	0.43%	−122,793	−48.1%
Hokuriku/Tokai	78,481	0.56%	51,477	0.37%	−27,004	−34.4%
Kinki	165,915	0.98%	85,083	0.52%	−80,832	−48.7%
Chugoku	90,041	1.64%	37,706	0.71%	−52,335	−58.1%
Shikoku	38,411	1.32%	19,162	0.69%	−19,249	−50.1%
Kyushu	127,879	1.23%	77,941	0.77%	−49,938	−39.1%
Total	967,753	1.04%	506,830	0.55%	−460,923	−47.6%

[a] Data for the year 2000 were extracted from a previous survey of hepatitis virus infections in Japan [5].
[b] The carrier rate specific for respective jurisdiction area was applied.

Discussion

There are many constraints in estimating total HCV and HBV infections in a given nation. Since it is not feasible to test every member for serological markers of hepatitis virus infection, populations representative of the entire nation have served for the estimation. Volunteer blood donors are recruited, but they have a restricted age range (16–64 years in Japan). Students attending schools and universities can close the opening in younger generations, but infants younger than the school age are not enrolled. Moreover, there are no means of estimating carrier rates of hepatitis virus infections in the individuals aged beyond the eligibility of blood donation. In addition, blood donors are selected individuals who are leading healthy lives above the average. In the survey of inhabitants in sentinel counties of the USA [6], who represent the average Americans, patients with liver disease and persons with restricted activities, such as those incarcerated or institutionalized, are not included.

Patients with clinical liver disease, as well as individuals found with HCV or HBV infection by health check-ups, can receive the medical care. However, many blood donors found with viral infections have developed severe liver disease already, and therefore, cannot receive efficient medical interventions [7, 8]. Hence, it is necessary to detect undiagnosed HCV and HBV infections hidden in the society. For this purpose, periodical health check-ups for screening hepatitis virus markers were started in April 2002 on the individuals, who turned 40, 45, 50, 55, 60 and 70 years, by a 5-year national project in Japan. The target age range (40–70 years) was selected due to a high incidence of hepatocellular carcinoma [9]. Since by far the majority of the first-time blood donors were younger than 40 years, the prevalence of HCV or HBV beyond that age dispersed widely (fig. 1, 4). In this study, therefore, the coverage by the first-time blood donors was confined to 20–39 years of age, and it was taken place by examinees of health check-ups aged 40–74 years; they left age groups ≤15 and ≥75 years uncovered, however.

The national prevalence of hepatitis virus infections in individuals ≤19 years was presumed to be similar to that in the Iwate prefecture situated in northern Japan. Since the prevalence of HCV or HBV infection in them was extremely low and stayed between 0.01 and 0.02%, such an assumption would not have affected the overall results to any significant extent. The prevalence of HCV in age groups ≥75 was simulated by a premise that it would be an exponential function of the age. Consequently, the formula based on profiles in five age groups from 50 to 74 years (at a 5-year notch) was extrapolated to three age groups ≥75 years. The simulation matched closely with the prevalence determined in corresponding age groups, with R^2 values ranging from 0.83 to 0.99 ($p < 0.05$ and $p < 0.01$, respectively) throughout 8 jurisdiction areas in Japan (fig. 3).

Japan has an axis spanning 2,000 kilometers from the north-east towards the south-west over the four major lands (Hokkaido, Honshu, Shikoku and Kyushu). Within a rather small land, the prevalence of HCV or HBV is not uniform all over Japan. The prevalence of HCV had an increasing gradient from north to south, and was the highest in Kyushu (table 2), while that of HBV was the highest in Hokkaido, decreased in between and then increased towards Kyushu (table 4). Reflecting such local differences, age-specific prevalence of HCV or HBV differed widely among 8 jurisdiction areas (fig. 2, 5).

Based on the results obtained on the area- and age-specific prevalence of HCV or HBV, carriers of these hepatitis viruses in 8 jurisdiction areas were tabulated separately over age groups from 20 to 74 years. Those in age groups ≤19 years were represented by the Iwate prefecture. The prevalence of HCV in age groups ≥75 years was simulated by the formula, and that of HBV was represented by individuals aged 70–74 years. Japan was populated by 127,767,994 people in 2005. Of these, 807,903 (95% CI 679,886–974,292) were estimated to have undiagnosed HCV infection at an overall prevalence of 0.63%, and 903,145 (837,189–969,572) to possess undiagnosed HBV infection at that of 0.71%. These estimates are much less than publically inferred numbers of HCV and HBV carriers in Japan at 1.5–2.0 million each. Leaving aside HCV and HBV carriers who have developed liver disease and stayed outside the scope of the present study, our estimates based on reasonable scientific grounds are much smaller; they add up barely half of generally referred figures around 1.5–2.0 million in Japan.

Based on the sex- and age-specific prevalence of hepatitis virus markers in the 3,478,422 first-time blood donors during 2001–2006, with the same criteria used in the previous study [5], we have estimated the number of undiagnosed HCV carriers aged 15–69 years in the year 2005 to be 398,567 (95% CI 295,410–501,453) and that of undiagnosed HBV carriers to be 506,830 (95% CI 398,115–616,113). In the previous study [5], undiagnosed HCV and HBV carriers aged 15–69 years in the year 2000 were assessed to be 884,954 (95% CI 725,082–1,044,826) and those with HBV to be 967,753 (95% CI 806,760–1,128,745). They decreased by 55.0 and 47.6%, respectively, during 5 years (table 5). In support of this view, the incidence of HCV or HBV infection during 10 years (1994–2000) in Japan is very low and estimated at 1.86 (95% CI 1.06–3.01) or 2.78 (1.87–4.145) per 100,000 person-years [10]. Decreases in undiagnosed HCV and HBV carriers in Japan would have been attributed to increased chances of receiving tests for hepatitis virus infections at health check-ups and medical institutions, as well as increased awareness due to educational programs or other healthcare campaigns or screening programs in high-risk individuals. Additionally, there would have been a cohort effect in individuals aged 15–69 years who have shifted by 5 years during the observation period.

The results of the Third National Health and Nutrition Survey (HANES III, 1988–1994) [11] and those of more recent HANES (2001–2002) [6] in the USA are essentially similar with respect to age-specific profiles of HCV infection, and shifted by 10 years. The incidence of de novo HCV and HBV infections may have decreased substantially both in the USA and Japan, driven partly by the introduction of the nucleic acid amplification test and a more stringent questionnaire on donors to exclude blood donations in the window period of infection [12–17]. The national burden of HCV infection has been reported in Great Britain [18], where the prevalence of anti-HCV in hospitalized patients was 3.4% and that in the first-time blood donors was 0.03% in the year 2008.

In spite of many improvements in the control of hepatitis virus infections, there are many HCV and HBV carriers buried in the society who need immediate identification for receiving timely and efficient medical interventions. Treatment of viral hepatitis keeps improving, especially for liver disease induced by HCV. The sustained virological response in the patients infected with HCV of genotype 1, who have received triple therapy with pegylated interferon, ribavirin and protease inhibitors, has increased to 70% or higher, from 50% with the state-of-care therapy with pegylated interferon and ribavirin [19, 20]. With the advent of new antiviral drugs that will enter the scene in the foreseeable future, the virological response is expected to increase further. There would be nothing like early detection of HCV and HBV infections for appropriate and timely medical care to prevent the progression of liver disease. Such a rational strategy will benefit not only patients themselves, but also merit the society and government, which are going to be burdened with ever-increasing morbidity and mortality along with skyrocketing costs.

Acknowledgements

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別紙様式第2-1
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医薬品
医薬部外品　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2011年8月10日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①②乾燥抗HBs人免疫グロブリン ③ポリエチレングリコール処理抗HBs人免疫グロブリン		研究報告の公表状況	Vox Sanguinis 2011;:1-2	公表国 アメリカ,欧州,東南アジア	
販売名（企業名）	①ヘブスブリン筋注用200単位　（ベネシス） ②ヘブスブリン筋注用1000単位　（ベネシス） ③ヘブスブリンIH静注1000単位　（ベネシス）					

研究報告の概要

我々は血漿由来医薬品の製造に使われる血漿分画プールのHEV RNAと抗HEV IgGを調査した。血漿プール(n=75)は欧州、北米、中東及びアジアから入手した。プールの約10%はHEV RNA陽性で、広範囲な地理的分布を示した（陽性プール：北米(n=1)、西欧(n=2)、東欧(n=1)、アジア(n=4)）。中東からのプールでは陽性サンプルは見つからなかった。陽性プールの何れもHEV RNA量が＞1000コピー/mLを超えていなかった。プールで見つかった低ウイルス量は、最初のクリオプレシピテートの後、更なる処理工程が行われる血漿由来凝固因子の何れの最終製剤からも、なぜMordow他がHEV RNAを検出しなかったかを説明するだろう。

別の可能性のある要因は、研究所間で大きく異なることが分かっているHEV RNA NAT分析法の感度であり、これは現在標準化されていない。献血者のHEVウイルス血症は10^7HEV RNAコピー/mLを超える可能性があり、従って、製造時に使われるプール・サイズとウイルス除去工程次第であるが、幾つかの血漿分画製剤でHEV RNAを検出できるかもしれない可能性はある。

プールで確認されたHEV株の系統樹解析（GenBank、受入番号：JN257704－JN257711）は、欧州と北米のプールで遺伝子型3ウイルスが見つかり、一方アジアのプールは地域で多くみられる遺伝子型4ウイルスを含んでいた。全配列は全てが他と異なっていた。プールで確認された2つの遺伝子型は、ヒトと共にブタのような動物の両方で見つかり、それは幾つかの症例における人獣感染の可能性を示唆した。

プール中の抗HEV IgGの存在は、MP BiomedicalsとAxiomの酵素免疫測定法を使って測定した。アジアのプールだけがMP Biomedicalsの測定系のカットーオフより大きい抗HEV IgG量を持っていることが分かった、そして結果はAxiomキットを使って確かめられ、地域の抗HEV抗体陽性率を反映した。抗HEVが幾つかのプールで特定されたものの、これが潜在的ウイルスの感染能の中和と関連するのかについては未だ明らかではない。最近の研究では、抗HEVを含むウイルス血症血清を使った培養で感染性HEVを増殖させることが可能であった。HEVは輸血によって伝播したが、プールした血漿によるHEV伝播の報告はない。HEVはナノろ過（＜20nm）によって除去できる、そしてそれはおそらく加熱処理によって不活化されると思われるが、その効果は不活化の条件や組成に依存している。しかし、抗HEV抗体量が低い地域では、HEVなどのノン・エンベロープウイルスに対する有効な除去工程を有さないSD処理血漿については、血漿プールのHEV RNAを検査するのが賢明かもしれない。

使用上の注意記載状況・その他参考事項等

代表としてヘブスブリンIH静注1000単位の記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体陰性であることを確認している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗HBs抗体を含有する血漿を原料として、Cohnの低温エタノール分画で得た画分からポリエチレングリコール4000処理、DEAEセファデックス処理等により抗HBs人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びウイルス除去膜によるろ過処理を施しているが、投与に際しては、次の点に十分注意すること。

4

報告企業の意見	今後の対応
HEVは直径27～38nmの球状粒子で、エンベロープはなく、長さ約7,300塩基対の一本鎖RNAを内包している。万一、原料血漿にHEVが混入したとしても、EMCおよびCPVをモデルウイルスとしたウイルスクリアランス試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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LETTER

Widespread distribution of hepatitis E virus in plasma fractionation pools

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Dear Editor,

We have investigated plasma fractionation pools, used in the manufacture of plasma-derived medicinal products, for HEV RNA and anti-HEV IgG. Plasma pools were obtained from Europe, North America, the Middle East and Asia. Approximately 10% of pools were positive for HEV RNA, showing a widespread geographic distribution (Table 1). Positive pools originated from plasma sourced in North America (n = 1), Western Europe (n = 2) and Eastern Europe (n = 1). No positive samples were identified in the pools from the Middle East. Of the Asian pools, four were positive for HEV RNA. None of the positive pools exceeded a load of >1000 copies/ml HEV RNA. The low viral loads found in pools may explain why Mordow *et al.* [1] did not detect HEV RNA in any final preparations of plasma-derived coagulation factors, which undergo further processing steps after initial cryoprecipitation. Another possible factor is the sensitivity of HEV RNA NAT assays that have been shown to vary widely between laboratories [2] and is not currently standardized. HEV viraemia in blood donors can exceed 7 $\log_{10}$ HEV RNA copies/ml [3]; therefore, dependent on pool size and virus reduction steps used during manufacture, it is possible that in HEV RNA might still be detectable in some plasma derivatives.

Phylogenetic analysis of the HEV strains identified in the pools (GenBank, accession numbers JN257704-JN257711) revealed that genotype 3 viruses were found in Europe and North America, whilst the Asian pools contained genotype 4 viruses that are more common in the region. All sequences were distinct from one other. The two genotypes identified in pools are found both in humans as well as in animal such as swine, with likely zoonotic transmission in some cases.

The presence of anti-HEV IgG in pools was determined using enzyme immunoassays from MP Biomedicals (MP Biomedicals Asia Pacific, Singapore) and Axiom (Axiom Diagnostic, Bürstadt, Germany). Only Asian pools were found to have anti-HEV IgG levels greater than the cut-off of the MP Biomedicals assay, and results were confirmed using the Axiom kit, reflecting the seroprevalence of anti-HEV in the region. Whilst anti-HEV is detectable in some pools, how this correlates to neutralization of potential virus infectivity remains unknown. In a recent study, it was possible to propagate infectious HEV in culture using viraemic serum containing anti-HEV [4]. There are no reports of HEV transmission by pooled plasma, although HEV has been transmitted by transfusion. HEV can be removed by nanofiltration (<20 nm), and it may be inactivated by heat treatment, the effectiveness of which is dependent upon parameters for inactivation and composition of the matrix [5]. However, where anti-HEV levels are low, solvent/detergent-treated plasma, with no effective reduction steps against non-enveloped viruses such as HEV, could present a risk for transmission, and it may be prudent to test such plasma pools for HEV RNA.

Table 1 Analysis of plasma fractionation pools for the presence of HEV RNA

Source of pools	No. positive/no. analysed
Europe	3/34
Europe/North America	0/3
North America	1/4
Middle East	0/11
Southeast Asia	4/23
Overall	8/75

RT-PCR was performed using total nucleic acid extracted using the COBAS AmpliPrep instrument (Roche Diagnostics GmbH, Penzberg, Germany). HEV RNA was detected using the One-Step RT-PCR kit (Qiagen GmbH, Hilden, Germany) using conserved primers forward 5′ GGG TGG AAT GAA TAA CAT GT and reverse 5′ AGG GGT TGG TTG GAT GAA) amplifying 233-bp products that were analysed by agarose gel electrophoresis and sequenced directly for genotyping purposes.

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医薬品
医薬部外品　　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2011 年 10 月 5 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①②ポリエチレングリコール処理抗破傷風人免疫グロブリン ③乾燥抗破傷風人免疫グロブリン	研究報告の公表状況	Vox Sanguinis 2011; Article published on line 29 SEP	公表国 イギリス		
販売名（企業名）	①テタノブリン IH 静注 250 単位　（ベネシス） ②テタノブリン IH 静注 1500 単位　（ベネシス） ③テタノブリン筋注用 250 単位　（ベネシス）					

研究報告の概要

英国における献血者のミニプール血漿中の E 型肝炎ウイルス RNA の検出

我々は（世界中の幾つかの地域から始まった）血漿分画プール中のE型肝炎ウイルス(HEV) RNAと抗体の検出を報告したベイリー等からの手紙を興味深く読んだ。著者等はプールの10%がHEV RNA陽性であった、そして血漿由来医薬品を使うことによる感染リスクを論ずるために報告した。
我々は最近、供血者パネル及び輸血に関連した伝播の可能性があるウイルス転換を示す英国とウェールズの献血者の現在のHEV感染の証拠を報告した。英国の血液供給への更なるHEVのリスクを把握するために、血清学的及び分子調査が2007年に集められた血漿ミニプールで行なわれた。それぞれのミニプールは48人の供血者から構成され、本来C型肝炎RNAスクリーニング様に準備したものだった。HEV RNAの抽出と検出は、前述した様に880のミニプール（約42,000人の供血者に相当）で実施された。880プール中の6つ(0.7%)がHEV RNAを検出可能であった。予想通り、HEV RNA陽性プール中のウイルス量は低かった（≦2000GEq以上/mL）。追加のHEV抗体（抗HEV）検査は、6つ全て(100%)で見つかった、そしてHEV RNA陽性プールの1/6(17%)はHEV IgGとIgMにそれぞれ反応した。検査した100のHEV RNA陰性プールの内、73%がHEV IgGに反応、HEV IgMは0%であった。
HEVによる無症候性感染の高い発生率は、献血者が受血者を感染させるに十分な機会を与える。一般的な英国人で行なわれた調査は13%以上の抗HEV血清陽性率を示し、年間6万人以上の症例が発生すると推定する。従って、我々の調査は検査したミニプール中の高い抗HEV IgG陽性率が示されたことに恐らく驚いていない。HEV RNAと抗HEV IgMの検出は、現在のHEV感染を示している。対照的に、英国の血清陽性率が与えた大変な驚きとして、ベイリー等はアジアからのプールだけでHEV IgGを発見した。彼らはまた、ヨーロッパからの検査したプール中のHEV RNAが8倍以上の高率であったことを報告したが、プールサイズを開示していない。これらの異いの幾つかは、プールの構成及び使われた検出分析のパラツキよって説明されるかもしれない。
まとめると、これらの報告は血液/血液成分と血液製剤からHEVを伝播する可能性の証拠を提供した。しかしながら、輸血後HEV伝播の範囲及びHEVを含んだ輸血製剤の受け入れの結果は、不十分な調査されていない。輸血関連のHEVリスクは免疫抑制した持続性HEVの著しい危害に関する新たなデータに照らして考慮に値する。英国の血液/血液成分の75%以上は、この集団に血液学的な支持として与えると推定する。従って、HEVと血液の安全性の課題は、更なる調査と議論が必要である。

報告企業の意見	今後の対応
HEVは直径27～38nmの球状粒子で、エンベロープはなく、長さ約7,300塩基対の一本鎖RNAを内包している。万一、原料血漿にHEVが混入したとしても、EMCおよびCPVをモデルウイルスとしたウイルスクリアランス試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

使用上の注意記載状況・その他参考事項等

代表としてテタノブリン IH 静注 250 単位の記載を示す。
2. 重要な基本的注意
1) 本剤の原材料となる血液については、HBs 抗原、抗 HCV 抗体、抗 HIV-1 抗体、抗 HIV-2 抗体陰性であることを確認している。更に、プールした試験血漿については、HIV-1、HBV 及び HCV について核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該 NAT の検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の破傷風抗毒素を含有する血漿を原料として、Cohn の低温エタノール分画で得た画分からポリエチレングリコール 4000 処理、DEAE セファデックス処理等により抗破傷風人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において 60℃、10 時間の液状加熱処理及びウイルス除去膜によるろ過処理を施しているが、投与に際しては、次の点に十分注意すること。

5

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LETTER

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Vox Sanguinis

Detection of hepatitis E virus RNA in plasma mini-pools from blood donors in England

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Dear Editor,

We read with interest the letter from Baylis *et al.*, [1] reporting on the detection of hepatitis E virus (HEV) RNA and antibody in plasma fractionation pools, which originated from several regions across the globe. The authors report that 10% of the pools were HEV RNA positive and discuss the transmission risk through the use of plasma-derived medicinal products.

We recently reported evidence of current HEV infection in English and Welsh blood donors indicating a turnover of the virus in the donor panel and the potential for transfusion-associated transmission [2]. To ascertain further the risk of HEV to the English blood supply, serological and molecular investigations were undertaken in plasma mini-pools collected in 2007. Each mini-pool was made up of 48 individual donors and had originally been prepared for hepatitis C RNA screening. Extraction and detection of HEV RNA was carried out on 880 mini-pools (equivalent to approximately 42 000 individual donors) as previously described [2]. Six of the 880 pools (0·7%) had detectable HEV RNA. As expected, viral loads in the HEV RNA-positive pools were low (≤ 2000 GEq/ml). Additional HEV antibody (anti-HEV) testing found all 6 (100%) and 1/6 (17%) of the HEV RNA-positive pools to be anti-HEV IgG and IgM reactive respectively. Of the 100 HEV RNA-negative pools tested, 73% and 0% were HEV IgG and IgM reactive respectively.

The high incidence of asymptomatic infection with HEV gives ample opportunity for blood donors to infect recipients. Studies undertaken in the general English population indicate an anti-HEV seroprevalence of ~13% and estimate that ~60 000 cases occur per year [3]. It is therefore perhaps unsurprising that our study demonstrates a high anti-HEV IgG prevalence in the mini-pools tested. The detection of HEV RNA and anti-HEV IgM demonstrates current HEV infections. In contrast, Baylis *et al.* [1] found HEV IgG only in the pools from Asia, which is very surprising given the UK seroprevalence. They also report ~eightfold higher rates of HEV RNA in tested pools from Europe but do not disclose the pool size. Some of these differences may be explained by variations in the make up of the pools and in the detection assays used.

Collectively, these reports provide evidence of the potential to transmit HEV from blood/blood components and products. However, the extent of HEV transmission post-transfusion and the outcome of receiving HEV-containing transfusion products remain poorly explored. The risks of transfusion-associated HEV deserves due consideration in light of emerging data on the significant harm of persistent HEV in the immunosuppressed [4, 5]. It is estimated that ~75% of UK blood/blood components are given as haematological support to this population. The issue of HEV and blood safety therefore warrants further studies and debate.

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一般的名称	①②ポリエチレングリコール処理抗破傷風人免疫グロブリン ③乾燥抗破傷風人免疫グロブリン					
販売名（企業名）	①テタノブリンIH静注250単位　（ベネシス） ②テタノブリンIH静注1500単位　（ベネシス） ③テタノブリン筋注用250単位　（ベネシス）	研究報告の公表状況	Transfusion（Transfusion）2011; 51(9): 1896-1908		公表国 アメリカ	

研究報告の概要

パルボウイルスB19(B19V)に対するレセプターが赤血球上にあるため、我々は試験管内のスパイク実験で血中のB19ウイルスの分布を調査し、自然感染におけるウイルスのコンパートメント化と持続性を評価した。二つの全血プロトコル（超遠心分離法のプロトコルと急速赤血球溶解/除去のプロトコル）は、定量リアルタイム・ポリメラーゼ連鎖反応を用いて評価した。全血に既知濃度のB19ウイルスがスパイクされ、様々な血液分画中における回収率が測定された。それから、急速赤血球溶解/除去のプロトコルが、「REDS 同種供血者と受血者の貯蔵庫（RADAR）」の凍結検体の中の43名のB19ウイルス感染ドナーから長期的に集められた104対の全血と血漿のペアにおけるB19ウイルス濃度の比較に使用された。

B19ウイルススパイク実験では、ウイルスDNAの約3分の1は血漿中で回収され、3分の2は赤血球に緩く結合していた。血漿B19ウイルスDNA濃度が100IU/mL以上の献血者の免疫グロブリン(Ig)M陰性期において、DNA濃度の中央値は血漿中よりも全血中で約30倍高かった。対照的に、IgM陰性時やB19ウイルスDNA濃度が低い時、全血対血漿比の中央値は約1であった。長期間の検体の分析は、全血中のB19ウイルスは持続的に検出されるが、血漿に対する全血のB19ウイルスの比は、血漿におけるウイルス量減少とIgM反応性低下を伴って減少することを明らかにした。

血漿に対する全血のB19ウイルスDNA比は感染ステージにより変化する：IgM陽性期では、全血では血漿の30倍のB19ウイルスDNA濃度があり、その後IgG抗体のみが存在する持続感染期にはその比は同等になる。このことが、B19ウイルスに感染した赤芽球由来のDNA陽性赤血球の存在と関係するか、B19ウイルスに特異的なIgM抗体を介したウイルスの細胞への結合と関係するかを決定するため、更なる研究が必要である。

使用上の注意記載状況・その他参考事項等

代表としてテタノブリンIH静注250単位の記載を示す。

1. 慎重投与
(1) 略
(2) 略
(3) 溶血性・失血性貧血の患者（ヒトパルボウイルスB19の感染を起こす可能性を否定できない。感染した場合には、発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。）
(4) 免疫不全患者・免疫抑制状態の患者（ヒトパルボウイルスB19の感染を起こす可能性を否定できない。感染した場合には、持続性の貧血を起こすことがある。）
2. 重要な基本的注意
(1) 略
1) 血漿分画製剤の現在の製造工程では、ヒトパルボウイルスB19等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。
6. 妊婦、産婦、授乳婦等への投与
妊婦又は妊娠している可能性のある婦人には、治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。（妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルスB19の感染の可能性を否定できない。感染した場合には胎

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報告企業の意見	今後の対応	児への障害（流産、胎児水腫、胎児死亡）が起こる可能性がある。）
ヒトパルボウイルスB19（human parvovirus B19：B19）は、脂質エンベロープを持たない極めて小さな（約20～26nm）DNAウイルスで、輸血や血漿分画製剤による伝播が報告されている。他のウイルスに比べて、血漿分画製剤の製造工程での不活化・除去が困難であり、本ウイルスの伝播リスクを完全に否定することはできないため、1996年11月より、使用上の注意にB19についての記載を行い注意喚起を図ってきた。万一、原料血漿にB19が混入したとしても、CPVをモデルウイルスとしたウイルスクリアランス試験成績及びB19を用いた不活化・除去試験の結果から、本剤の製造工程において十分に不活化・除去されると考えている。なお、原料血漿へのB19混入量低減のため、B19-ミニプールNATが米国の原料供給元で行われている。	本報告は本剤の安全性に影響を与えないものと考えるので、特段の措置はとらない。	

抗破傷風人免疫グロブリン

TRANSFUSION COMPLICATIONS

Distribution of parvovirus B19 DNA in blood compartments and persistence of virus in blood donors

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BACKGROUND: Because the receptor for parvovirus B19 (B19V) is on red blood cells (RBCs), we investigated B19V distribution in blood by in vitro spiking experiments and evaluated viral compartmentalization and persistence in natural infection.

STUDY DESIGN AND METHODS: Two whole blood (WB) protocols (ultracentrifugation and a rapid RBC lysis and removal protocol) were evaluated using quantitative real-time polymerase chain reaction. WB was spiked with known concentrations of B19V and recovery in various blood fractions was determined. The rapid RBC lysis and removal protocol was then used to compare B19V concentrations in 104 paired WB and plasma samples collected longitudinally from 43 B19V-infected donors with frozen specimens in the REDS Allogeneic Donor and Recipient Repository (RADAR).

RESULTS: In B19V spiking experiments, approximately one-third of viral DNA was recovered in plasma and two-thirds was loosely bound to RBCs. In the immunoglobulin (Ig)M-positive stage of infection in blood donors when plasma B19V DNA concentrations were greater than 100 IU/mL, median DNA concentrations were approximately 30-fold higher in WB than in plasma. In contrast, when IgM was absent and when the B19V DNA concentration was lower, the median WB-to-plasma ratio was approximately 1. Analysis of longitudinal samples demonstrated persistent detection of B19V in WB but declining ratios of WB to plasma B19V with declining plasma viral load levels and loss of IgM reactivity.

CONCLUSIONS: The WB-to-plasma B19V DNA ratio varies by stage of infection, with 30-fold higher concentrations of B19V DNA in WB relative to plasma during the IgM-positive stage of infection followed by comparable levels during persistent infection when only IgG is present. Further study is required to determine if this is related to the presence of circulating DNA-positive RBCs derived from B19V-infected erythroblasts, B19V-specific IgM-mediated binding of virus to cells, or other factors.

The understanding of the natural history of parvovirus B19 virus (B19V) infection is that in most immunocompetent individuals (e.g., blood donors), viremia occurs approximately 1 week after infection and persists at high titers in plasma for approximately 5 days.[1] Immunoglobulin (Ig)M antibody develops at approximately 12 days after infection and IgG antibody follows within days coinciding with precipitous declines in plasma viremia levels. Subsequently, plasma viremia disappears generally within weeks, IgM antibody becomes undetectable after several months (although this precise duration is unknown), whereas IgG antibody persists long term and is thought to convey immunity to reinfection. Recently it has become established that a variation of this natural history occurs in people in whom chronic persistent B19V infection occurs; this is characterized by low plasma levels of B19V DNA persisting for more than 6 months in conjunction with IgG antibody.[2-7]

The receptor for B19V on marrow red blood cell (RBC) progenitor cells is the P blood group antigen.[8,9] This receptor is also present at high concentrations on mature

ABBREVIATIONS: B19V = parvovirus B19; CBER = Center of Biologics Evaluation and Research; RADAR = REDS Allogeneic Donor and Recipient Repository; TC = target capture; VL(s) = viral load(s); WB = whole blood.

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circulating RBCs in almost all individuals, with the exception of rare persons with the null p phenotype. Binding of B19V to mature RBCs is known to occur and has been exploited in development of RBC B19V antigen agglutination assays.[10] Thus, it is theoretically possible that a substantial proportion of B19V in blood is adsorbed to or persists within RBCs from the infected erythroblast phase and that B19V DNA concentrations will consequently differ in plasma and cellular blood compartments. Also, it is unknown if the partitioning of B19V between plasma and cellular blood compartments varies during different stages of infection, possibly due to the effect of IgM and IgG antibodies on B19V particles enhancing or blocking binding to one or more cellular blood elements (e.g., RBCs, white blood cells [WBCs], or platelets [PLTs]).

B19V contamination of plasma derivatives has led to widespread adoption of B19V DNA screening of source and recovered plasma donations to interdict high-titer viremic units before pooling and fractionation.[11-13] Transfusion-transmitted B19V infection from blood component transfusion occurs infrequently but has been documented in several case reports, including a recent case in the United States.[14] Although screening of whole blood (WB) components intended for individual patient transfusions is not currently routinely performed (except in Germany, Austria, and Japan, where this screening is performed on plasma and targets units with high B19V DNA concentration), the issue of compartmentalization of B19V in blood could be important if policies evolve toward further testing.

This study's major objective was to establish the relative concentrations of B19V DNA in plasma versus WB and to determine if this "compartmentalization" varies in different stages of infection. To accomplish this, we developed procedures to apply a sensitive B19V polymerase chain reaction (PCR) assay to WB samples. This involved a series of in vitro spiking experiments to establish that 1) B19 viral standards contained intact viral particles that could be pelleted by our ultracentrifugation protocol; 2) spiking high-titer B19V standards into fresh and frozen WB to establish the partitioning of exogenously spiked B19V in various blood compartments; and 3) development of a special reagent to overcome the inability of the ultracentrifugation-target capture (TC) protocol (as demonstrated in our experiments) to reliably recover and detect low levels of B19V DNA in WB. After this in vitro experimental work, we then used our standard plasma TC-PCR and this novel WB protocol to test serial samples from donors with previously documented B19 plasma viremia whose samples were stored in the REDS Allogeneic Donor and Recipient Repository (RADAR). As a secondary objective, we also evaluated the rate of B19V DNA persistence in those B19V DNA–positive donors who had a repository donation that was given at least 6 months before or subsequent to their DNA-positive donation.

MATERIALS AND METHODS

Standard B19V assays

Plasma B19V PCR assay

This assay has previously been described in detail.[15,16] It is based on a magnetic-bead B19V DNA capture step followed by a TaqMan real-time PCR assay targeting the VP1 region of the Genotype 1 B19V genome. Assay sensitivity was established as a 50% limit of detection of 1.6 IU/mL (95% confidence interval [CI], 1.2-2.1) and a 95% limit of detection of 16.5 IU/mL (95% CI, 10.6-33.9). When run against a standard curve, the assay can also be used to quantify B19V DNA with the lower limit of quantitation at 20 IU/mL. Alternately, in in vitro spiking studies a difference in cycle threshold (i.e., the cycle at which DNA was initially detected) was used to compare relative quantities of B19V DNA in different blood compartments.

B19V antibody testing

Specimens were tested for the presence of B19V IgG and IgM antibodies against a recombinant VP2 protein with Food and Drug Administration (FDA)-licensed test kits (Biotrin, Dublin, Ireland). Due to sample volume considerations, testing was performed in singlicate (rather than in duplicate as stated in the package insert) by accessing a 0.25-mL subaliquot of plasma from the RADAR repository. If results fell into the equivocal zone, the assay was repeated in singlicate and the repeat result was taken as the overall final result for the specimen.[15,16]

Protocol for development of WB PCR assays and for in vitro spiking studies to compare levels of B19V in plasma versus WB compartments

B19V standard and spiked controls

We used a Genotype 1 B19V standard from the Center of Biologics Evaluation and Research (CBER, Rockville, MD) to prepare our spiking B19V preparation. The CBER B-19–positive standard[17] was from a window period plasma donation. We generated plasma and WB-spiked controls at serial twofold dilutions, with concentrations equal to 1000, 500, 250, 125, 62.5, and 31.25 IU/mL. These spiked standards were aliquoted and frozen at –80°C. Unspiked samples were also prepared as negative controls. These standards were used in both the ultracentrifugation and the WB processing (HemoBind, US patent application pending; http://www.faqs.org/patents/app/20100092980) protocols. To make a relevant and equitable comparison all the WB and plasma assays used in this study were designed to assay 0.5 mL per reaction, as was used in our previous study.

Validation of the ultracentrifugation protocol using plasma controls

Plasma spiking standards, as described above, were ultracentrifuged for 2 hours (4°C) at 50,377× *g* (Sorvall Stratos, Thermo Scientific, Asheville, NC) to pellet intact viral particles and thereby confirm that B19V DNA in the CBER standard was virion associated and that the ultracentrifugation protocol could efficiently recover all B19V in the standard. The supernatant was removed and the pellet digested with proteinase K (20 mg/mL) overnight; these samples were processed by B19V TC and amplified using our real-time PCR protocol. The results from the ultracentrifugation protocol were compared to the results generated using our standard assay procedure applied to corresponding spiked plasma preparations that were not subjected to ultracentrifugation and pellet extraction.

Ultracentrifugation protocol for frozen WB

Fresh WB, spiked with B19V to achieve a concentration of 100,000 IU/mL (derived from a high-titer plasma donation that tested negative for B19 IgM and IgG antibodies by the Biotrin assay), was incubated at room temperature for 1 hour and then aliquoted and frozen at –80°C. The frozen blood was thawed and mixed with an equal volume of RBC saponin lysis solution (0.4% saponin in 0.5% NaCl, pH 7.4) to completely lyse residual RBC in the already hemolyzed thawed-frozen WB. The preparation was ultracentrifuged at 50,377× *g* for 2 hours. The supernatant was transferred to a second tube, leaving the degraded RBC membranes and viral particles in a pellet in the primary tube. The pellet was subjected to protein digestion by adding 200 uL of an equal part of Solution A (0.1 M KCl, 0.01 M Tris Base pH 8.3, 0.0025 M $MgCl_26H_2O$) and Solution B (10 mM Tris, pH 8.3, 2.5 mM $MgCl_26H_2O$, 1% Tween-20, 1% NP40) mixed with 1.25 uL of proteinase K (20 mg/mL). Protein digestion was performed at 60°C, for 2 hours, vortexing vigorously every 15 minutes. Both pellets and supernatants were tested for B19V DNA by TC and real-time PCR amplification.

Separation of WB into compartments after incubation with B19V

Plasma containing B19 virions was spiked into freshly drawn WB to achieve a concentration of 100,000 IU/mL followed by incubation at room temperature for 24 hours. After incubation, the WB was centrifuged at 833 × *g* for 5 minutes to separate the plasma from the packed red cell (pRBC) compartment, which includes the buffy coat layer. The plasma collected at this point served as reference data for other compartments.

In a "without wash" experiment, after removal of plasma, the pRBCs were not washed and were directly subjected to RBC lysis followed by ultracentrifugation to generate a pellet composed of viral particles, RBC membranes, and WBCs/PLTs. In the "wash" experiment, after removal of plasma, the remaining pRBCs with buffy coat (WBCs/PLTs) were washed twice with PBS to ensure that viral particles that were not tightly bound to cells were washed away before proceeding with RBC lysis using a Saponin RBC lysis solution. After lysis, the preparation was centrifuged at 4000 × *g* for 5 minutes, generating a hemolyzed RBC supernatant and a pellet of WBCs and large PLTs. The supernatant, containing lysed RBC membranes and presumably bound viral particles, was collected into a tube and subsequently ultracentifuged. The pellet was washed again to ensure no residual lysis solution remained. The RBC supernatant and the wash solutions were ultracentrifuged at 50,377 × *g* (Sorvall Stratos, Thermo Scientific) for 2 hours to bring down viral particles, RBC membranes, and viral particles bound to the membranes. The pellet and the supernatant gathered after ultracentrifugation were collected for testing. The RBC membrane pellets from the ultracentrifugation and the WBC and PLT pellets were subjected to protein digestion. B19V DNA was extracted from all compartments using TC and amplified using real-time PCR.

Novel RBC lysis and removal protocol for WB preparations

To address the problem of processing WB for detection of both plasma and cell-associated nucleic acids without use of ultracentrifugation, we developed a new method for rapid RBC lysis and removal of hemoglobin (Hb) and other possible inhibitors of TC and PCR processing (HemoBind WB processing protocol). An equal volume of 6 molar guanidine-HCl-0.2 mol/L ethylenediaminetetraacetat lysis buffer (pH 8.0) was added to 0.5 mL of frozen-thawed WB. The sample was vortexed and incubated at room temperature while gently mixing in a rocker. The sample was pulse spun at high speed in a microcentrifuge to pellet particulate material; all cell- and plasma-derived nucleic acids would be expected to remain in the guanidine lysate supernatant. One milliliter of HemoBind buffer was added to 1 mL of the lysate supernatant, along with 20 µL of proteinase K (20 mg/mL). The mixture was vortexed and incubated at 60°C for 30 minutes, and then clarified of precipitates by centrifugation in a high-speed microcentrifuge for 1 minute. The supernatant was transferred to a clean mirocentrifuge tube and heated at 100°C for 5 minutes. The preparation was microcentrifuged at high speed for 1 minute and the clear supernatant was transferred to the 10-tube units used in the Procleix TC system. The protocol for plasma TC was followed. As with the plasma protocol, the WB samples were amplified with internal controls. Results from samples with positive internal controls were deemed valid. Valid results with less or equal to cycle threshold of 40 were considered positive and those with amplifications greater than cycle threshold of 40 were considered negative.

Selection of paired plasma and WB samples from the RADAR repository

The National Heart, Lung, and Blood Institute RADAR repository was established to investigate possible transfusion-transmitted infections and has been described in detail elsewhere.[18] Repository specimens were collected from 2000 through 2003 by blood centers and selected hospitals at seven geographically dispersed US locations. All enrolled subjects gave informed consent for frozen storage of their specimens and for subsequent testing of these specimens for possible transfusion-transmissible infections. There was no restriction on how many times a repeat donor could be recruited to give a repository sample.

The donor portion of the repository consists of 13,201 donation specimens (given by 12,408 distinct donors) that were transfused to enrolled recipients and 99,906 donation specimens (contributed by 84,339 donors) from donations that were not transfused to enrolled RADAR recipients. Repository specimens consisted of two frozen 1.8-mL plasma aliquots and a 1.5-mL sample of frozen WB.

In previous studies, B19V DNA and antibody testing had been performed on plasma samples from 17,549 donor repository samples (5020 from donors without enrolled recipients and 12,529 from donors with enrolled recipients).[15,16] As previously reported, this testing identified 149 samples from 147 donors that tested positive for B19V DNA in plasma. For this study, we searched through the RADAR repository records to determine whether any of the donors with positive PCR results had made additional repository donations which had not yet been evaluated for B19V DNA. We found that of the 149 PCR-positive donors, 43 had samples from more than one blood donation included in the repository. In total, there were 137 donations given by these 43 donors. The index samples (i.e., those previously selected for B19V analysis), the immediate previous and subsequent donations, and the first and last donation in donors with greater than three donations from each of these 43 donors were selected for this study; additional intervening donations were also tested in selected cases. Thus, a total of 104 donations were tested by plasma B19V PCR, WB B19V PCR using the HemoBind protocol, and B19V IgM and IgG antibody assays. Quantitative results from plasma and WB PCR assays were obtained as two separate replicate determinations with results reported as the mean of these results. Personnel performing the study testing were kept blinded as to the identities of the paired WB and plasma samples. For the 43 index donations, we used the plasma B19V PCR and antibody results from previous studies rather than performing these tests again due to limitations in sample volumes.

We constructed a ratio of B19V DNA concentration in WB divided by B19V DNA concentration in plasma. Samples with positive PCR results that were below the quantitative detection limit of 20 IU/mL B19V DNA were assigned a value of 10 IU/mL.

Donation and/or donor classification

A donor was classified as having chronic persistent infection if two consecutive samples separated by 6 or more months were B19V DNA positive. A donor was classified as recently infected if a donation was IgM antibody positive or if a positive DNA result occurred in a donation given subsequent to a donation that tested B19V DNA, IgM, and IgG negative. A donor was classified as a remote infection if neither of the above criteria applied and if the DNA-positive donation was also IgG positive.

Statistical analysis

B19V DNA concentrations in paired WB and plasma samples were compared using the sign test. The median (and 95% CI) ratio of B19V DNA in WB and plasma was determined. A nonparametric regression of B19V DNA in WB and plasma was performed.

RESULTS

Recovery of B19V DNA from spiked plasma and WB by ultracentrifugation

Quantitative standard curve plasma controls from a previous study were used to validate an ultracentrifugation protocol designed to recover B19 viral particles from plasma and WB preparations. Spiked plasma controls were ultracentrifuged and resulting pellets (which should contain all intact viral particles but not soluble DNA) and supernatant were each tested to evaluate the efficacy of viral DNA recovery. Depending on the dilution assayed, the cycle threshold of the amplification curve derived from the pellet was 6 to 16 cycles earlier than for the supernatant. Since each PCR cycle threshold (C_T) difference corresponds to a twofold difference in starting concentration of the target nucleic acid, these ΔC_T results indicate that the B19 virion particle–associated DNA recovery using the ultracentrifugation protocol was greater than 98% (i.e., only $1/2^6$ to $1/2^{16}$ of B19V DNA remained in the supernatant), thereby establishing the ability to recover virtually all B19 viral particles from plasma samples spiked with B19V standards. Of note, the C_T values observed after amplification of ultracentrifuge pellets derived from each concentration of spiked virus were similar to those of our previous study, in which the spiked plasma samples were directly subjected to the same TC and real-time PCR assay (Table 1 and Kleinman et al.[16]).

Frozen WB samples spiked with 100,000 IU/mL of B19 virions were then tested to determine the recovery of B19V DNA by the ultracentrifugation protocol. The C_T values of

TABLE 1. Recovery of spiked B19V DNA spiked into plasma using the ultracentrifugation protocol

B19V DNA spike (IU/mL)	Pellet C_T	Supernatant C_T	ΔC_T	% recovery in pellet
1000	30.5	37	6.5	98.9
500	31.5	41	9.5	99.8
250	32.5	40	7.5	99.5
125	33	39	6	98.4
62.5	34	50	16	100

real-time PCR amplifications were compared between the ultracentrifuge pellets and supernatants. In four experiments, the ΔC_T between the pellet (C_T = 22, 26, 24, 20) and supernatants (C_T = 28, 33, 50, 30) were 6, 7, 16, and 10, respectively, indicating B19 viral or DNA recoveries of greater than 98%, similar to the results with comparably spiked and processed plasma controls.

B19V DNA recovery from blood compartments following in vitro spiking of WB

We incubated fresh WB with high-titer B19V–positive plasma from a donor in the acute preseroconversion phase of infection to achieve a final concentration of 100,000 IU/mL WB, thereby simulating a blood specimen acutely infected with B19V. After incubation for 24 hours, WB was processed into three compartments: plasma, RBCs, and WBCs and/or PLTs. The B19V DNA levels were quantified in each of these compartments using sample processing, ultracentrifugation, and TC real-time PCR protocols detailed under Materials and Methods.

The results are presented in Fig. 1. Plasma was used as the reference compartment, and hence data from plasma were plotted as 1.0 in both Fig. 1A and Fig. 1B, with levels of B19V DNA derived from the other compartments plotted as ratios to the corresponding plasma data. The data in Fig. 1A were generated without a wash step and therefore do not include results for "first PBS wash" and "second PBS wash." The concentration of B19V DNA derived from the RBC and WBC and/or PLTs compartments was more than twice the level derived from plasma; in other words, the plasma B19V DNA concentration was less than one-third of the total B19 virus or DNA that had been spiked into WB.

The data in Fig. 1B were generated using a slightly modified protocol incorporating washing of cellular (RBCs and WBC and/or PLTs) preparations before proceeding with lysis of RBCs, pelleting, and B19V DNA extraction. In this protocol, the cells were washed twice with PBS and the amplification results of washing solution specimens were plotted as "first PBS wash" and "second PBS wash." Added together, these two washes yielded about twice the B19V DNA as did plasma. After being washed twice with PBS, very little B19V DNA was recovered in pellets derived from the RBC and WBC and/or PLT compartments. This indicates that after in vitro spiking, approximately two-thirds of B19 virus or DNA is associated with cellular constituents and one-third with plasma, similar to the results derived with the ultracentrifugation protocol using spiked WB preparations, but that this binding is weak and reversible.

Validation of HemoBind and TC protocol

For the in vitro spiking experiments reported above, high concentrations of virus (100,000 IU/mL) were spiked into WB samples to show compartmentalization of the virus. However, this input level is much higher than the concentrations of B19V that we have observed in RADAR donation samples[15,16] and also beyond the limits of our standard curve. When the ultracentrifugation protocol was applied to WB spiked with the standard curve controls, the recovery at 1000 IU/mL was the same for plasma and WB, but the recovery in WB samples decreased and became variable with decreasing viral input compared to the plasma samples (Fig. 2). The WB spiked with the 62.5 IU/mL control yielded poor and erratic recovery of virus (standard deviation ± 4.8 cycles), while the WB spiked with 31.25 IU/mL control yielded no detectable B19V DNA after ultracentrifugation and TC-PCR. Because the ultracentrifugation protocol had low viral recovery at the low end of the WB standard curve, we created a new protocol using HemoBind to process WB samples to enhance the sensitivity of the assay.

Frozen WB standard controls (spiked and nonspiked) were processed using HemoBind to eliminate cellular debris and potential inhibitors of TC and real-time PCR. After HemoBind was added into frozen WB and a clarified solution obtained, the samples were processed by TC and real-time PCR in parallel with plasma standard controls. In Fig. 2, the C_T of real-time PCR amplifications were compared between HemoBind-processed WB samples and the corresponding plasma samples processed using the standard protocol. The slopes for the three procedures (TC and real-time PCR on plasma [TC-RT plasma], the HemoBind and TC real-time PCR on WB [HB-TC-RT WB], and the ultracentrifuged and real-time PCR [UltCfg-RT] on plasma) were −0.99, −0.98, and −1.00, respectively, very close to the theoretical slope of −1.00. The intercepts of TC-RT plasma and HB-TC-RT WB were 39.1 and 39.6, respectively. The similar slopes and intercepts indicate minimal difference between the two protocols, with differential C_T's of only approximately 0.5 cycles.

Comparison of B19V DNA in paired WB and plasma samples from blood donors

Figure 3 summarizes the WB relative to plasma concentrations of B19V for all 104 samples with positive B19V DNA

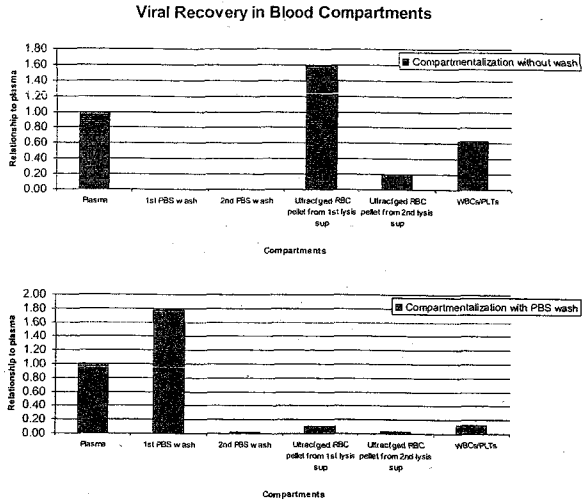


Fig. 1. Distribution of B19V DNA in compartments of WB samples after incubation with B19 virus stock at 100,000 IU/mL. The relative concentrations of recovered B19V DNA are plotted on the *y*-axis using the concentration in the plasma sample as a reference with a designated value of 1.00. The *x*-axis shows the different compartments prepared from the WB samples. Less than one-third of total B19V DNA was present in plasma.

results. Concentrations in WB samples tended to be higher than concentrations in plasma samples ($p = 0.0005$, sign test). For the total sample set, WB samples yielded 1.9-fold higher B19V DNA concentrations than their paired plasma samples (median WB-to-plasma ratio, 1.9; 95% CI, 1.2-4.2). This ratio varied throughout the range of plasma B19V DNA load; for plasma samples with B19V DNA concentrations of greater than 100 IU/mL, this median ratio was 19.5, whereas the ratio was only 1.9 for samples with plasma B19V DNA concentrations of greater than 20 to 100 IU/mL. While the WB-to-plasma ratio for the complete data set is significantly greater than 1, the ratio varies in samples with much higher ratios observed among donor specimens with high plasma B19V DNA levels ($p = 0.01$ by nonparametric regression goodness-of-fit test).

B19V DNA WB-to-plasma ratios at different infection stages

In Fig. 4, B19V DNA WB-to-plasma ratios for 74 blood donations with quantitative (e.g., nonzero) WB and plasma DNA results were analyzed relative to the IgM and IgG antibody status of the donation, which reflects the stage of B19V infection. For 20 IgM-positive, IgG-positive recent infections, the mean ratio was 66.1, with a median of 17.4 and a range of 0.8 to 824.3. For 54 remote infections (IgM negative, IgG positive), this mean ratio was 15.4 with a median of 1.5 and a range of 0.06 to 657.0. The difference in WB-to-plasma B19V DNA ratios between these two stages of infection was significant ($p \leq 0.001$, Mann-Whitney rank-sum test).

Table 2 provides a composite analysis of B19V DNA WB-to-plasma ratios classified by both plasma DNA concentration and IgM status. The highest median ratio (29.7) was found in IgM-positive donations with B19V DNA of greater than 100 IU/mL.

Stage of B19V infection and persistence in blood donors

Figure 5 plots data for the donors with multiple donations from which paired WB and plasma samples were available in the RADAR repository. Figure 5A presents detailed quantitative DNA and antibody results from one representative B19-viremic blood donor with recent infection (IgM positive) that evolved to chronic persistent infection. This donor had repository samples from donations made during both the early and the later stages of B19V infection

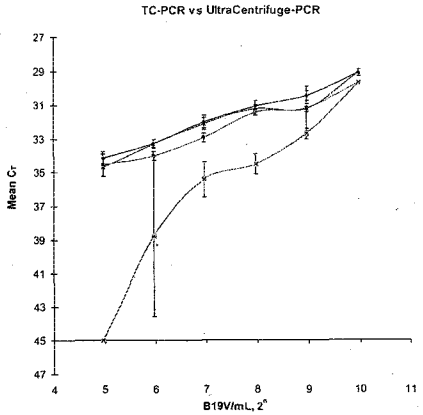


Fig. 2. Viral recovery from ultracentrifugation and HemoBind TC protocols. The *y*-axis shows mean C_Ts ($n = 4$) with ±1 SD error bars of real-time PCR amplification. The B19 viral input is plotted on the *x*-axis in base 2 log (2^n) format since PCR amplicons theoretically double in quantity per amplification cycle. The control standards are serial twofold dilutions (1000, 500, 250, 125, 62.5, and 31.25 IU/mL). TC-HemoBind WB (■) refers to the protocol using HemoBind, TC, and real-time PCR amplification. TC-Plasma (◆) refers to the protocol using TC and real-time PCR amplification for plasma. UltCfg-Plasma (▲) and UltCfg-WB (✱) refer to protocols using ultracentrifugation and real-time PCR amplification.

as evidenced by serial donation samples with decreasing IgM reactivity in parallel with increasing IgG seroreactivity. Note that the B19V DNA loads of WB samples at the early stage of infection were significantly higher than those of their paired plasma samples, as indicated by the WB-to-plasma B19V DNA ratios at the top of the figure. In contrast, at the later stages of infection, the WB and plasma B19V DNA levels were almost equal.

Figure 5B shows results for 29 donors whose donations were separated by at least 6 months. Of these, 25 made at least one donation at a greater than 6-month interval from their initial DNA-positive donation. Based on plasma B19V DNA detection, 22 (88%) showed chronic persistent infection; this percentage fell to 72% (18 donors) with the WB assay. Five of the persistently infected donors had additional test results at more than 2 years at which time four donors were B19V DNA negative on both their plasma and their WB samples. All donors with detectable IgM showed loss of the antibody over time. The 14 additional donors (not shown in Fig. 5B), with less than 6 months of follow-up, showed an even higher rate of persistence over the short time frame in which they were evaluated.

Four donors in this study were documented to have incident infection, characterized by a donation that was negative for B19V DNA and antibody followed by a sample that was DNA positive, IgG positive, and in three of four cases, IgM positive (Fig. 5B, and not shown). An additional 12 donors had recent infection based on the detection of IgM antibody in the initial PCR-positive donation (eight cases shown in Fig. 5B; four cases not shown). Three donors with B19V IgG antibody but without detectable B19V DNA on their initial donation showed detectable low-level B19V DNA on their follow-up donation; this may represent either intermittent low-level viremia or a limitation of our assay in consistently detecting such low B19V DNA levels (Fig. 5B, and not shown). Finally, we found one antibody-negative donor with a low level of B19V DNA in both plasma (132 IU/mL) and WB (15 IU/mL); on a subsequent donation given almost 6 months later, this donor tested B19V DNA negative but did not demonstrate antibody seroconversion (case not plotted in Fig. 5B).

DISCUSSION

Because B19V is known to infect and replicate in erythroblasts in the marrow and because mature RBCs contain the B19V receptor (i.e., the P antigen) on their cell surface,[19] we speculated that B19V may be within or bound to circulating erythroid cells and hence viral DNA concentrations might differ in WB and plasma. In addition, previous work with other viral agents had demonstrated that for some viruses that exhibit significant plasma viremia (e.g., human immunodeficiency virus [HIV], hepatitis C virus, West Nile virus, Dengue), a substantial proportion of viral nucleic acid in blood is found to be cell associated when appropriate WB-based nucleic acid test (NAT) is performed.[18-23] Potential mechanisms to explain this finding are the presence of intracellular viral nucleic acid due to active replication in cellular compartments (e.g., HIV DNA and RNA in CD4+ T cells) and/or binding of viral particles to cellular constituents of blood, including PLTs (for HIV and Dengue)[19-22] and RBCs (for HIV and WNV).[23,24]

Before evaluating cases of natural B19V infection, we began our study by evaluating different laboratory protocols for B19V WB NAT. WB NAT assays are challenging due to the fact that Hb and other cell-derived constituents inhibit nucleic acid amplification.[25] This applies both to PCR assays (which are inhibited by as little as 1.3 µg/mL of Hb) and to the transcription-mediated amplification assay method commercially developed by Gen-Probe (San Diego, CA). In this latter assay, WB inhibition occurs both at the TC and amplification steps.[26,27] Although a 5- to 10-fold dilution of WB has been demonstrated to overcome this interference, this dilution results in a loss of assay sensitivity.[26,27]

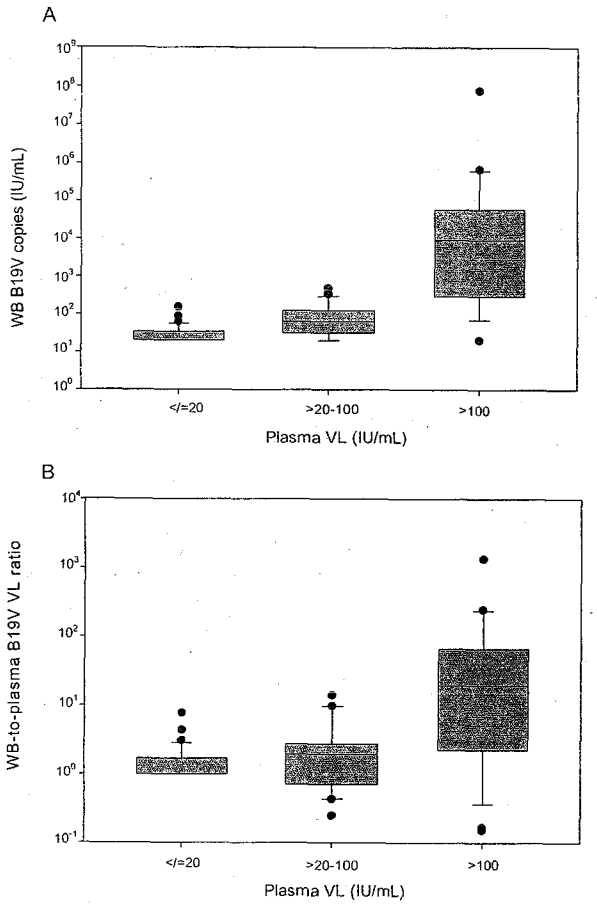


Fig. 3. (A) Box-and-whisker plot of B19V VLs in the WB compartment compared to corresponding plasma VLs. When plasma VL was positive but not more than 20 IU/mL (n = 58), the mean VL in WB was 27.54 IU/mL (median, 20 IU/mL). In the midrange of VL (>20-100 IU/mL, n = 24), the mean WB VL was 100.63 IU/mL (median, 63.2 IU/mL). At the high end, when the plasma VL was greater than 100 IU/mL (n = 22), the mean WB VL was 3.48×10^6 IU/mL (median, 9199 IU/mL). (B) WB-to-plasma VL ratio relative to plasma VL. At the low end of the plasma VL distribution, the WB-to-plasma ratio ranges from 0 to 7.66, with a median of 1 and mean of 1.38. In the midrange (>20-100 IU/mL, n = 24), the WB-to-plasma ratio range was 0.25-13.90, with a median of 1.95 and mean of 2.83. When the plasma VL was greater than 100 IU/mL, the WB-to-plasma ratio range was from 0.15 to 1332, with a median of 19.47 and mean of 103.52.

In this study we evaluated whether ultracentrifugation of blood lysates could quantitatively recover viral particles and overcome these inhibitory effects. We demonstrated that our ultracentrifugation protocol was effective at recovering B19V when high concentrations of B19V were spiked into plasma or WB. Using this protocol, we established that less than one-third of B19V spiked into WB was present in plasma, with the remainder in the cellular compartments and primarily in the RBC compartment.

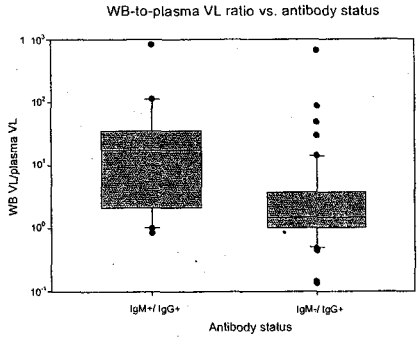


Fig. 4. Box-and-whisker plot of the B19V DNA ratios of WB samples to plasma samples based on IgM and IgG status, IgM positive and IgG positive (IgM+/IgG+; n = 20) and IgM negative and IgG positive (IgM−/IgG+; n = 54). The B19V DNA ratios are plotted on the *y*-axis and the serologic status is plotted on the *x*-axis. (p > 0.001, Wilcoxon rank-sum test).

When this cellular compartment was subjected to serial low-speed centrifugation and washing steps, most of the initially bound virus was present in the eluate rather than retained in the cellular preparation. We speculate that the explanation for these results is loose binding of spiked B19V to RBCs, either through weak binding to the P blood group receptor site or through nonspecific binding. These findings are not inconsistent with development of B19 antigen agglutination assays employed in donor screening in Japan, given that those assays employ optimized conditions to maximize binding and they require more than 10^{10} B19 particles/mL of plasma for positive results.[28,29]

The above spiking and recovery studies were performed using a high concentration of B19V. Further experiments demonstrated that the ultracentrifugation protocol was not efficient in recovering B19V when lower concentrations of virion particles were spiked into WB. We speculate that the difference in the protocol efficiency at high and low viral spiking concentrations was due to the need to have sufficient concentrations of virus to create ultracentrifugation pellets that could withstand subsequent washing and recovery procedures. Use of unrelated carrier virus particles or microbeads to facilitate B19V recovery at low spiking levels could potentially overcome the sensitivity problem of the ultracentrifugation protocol (as discussed in Lee et al.[20]).

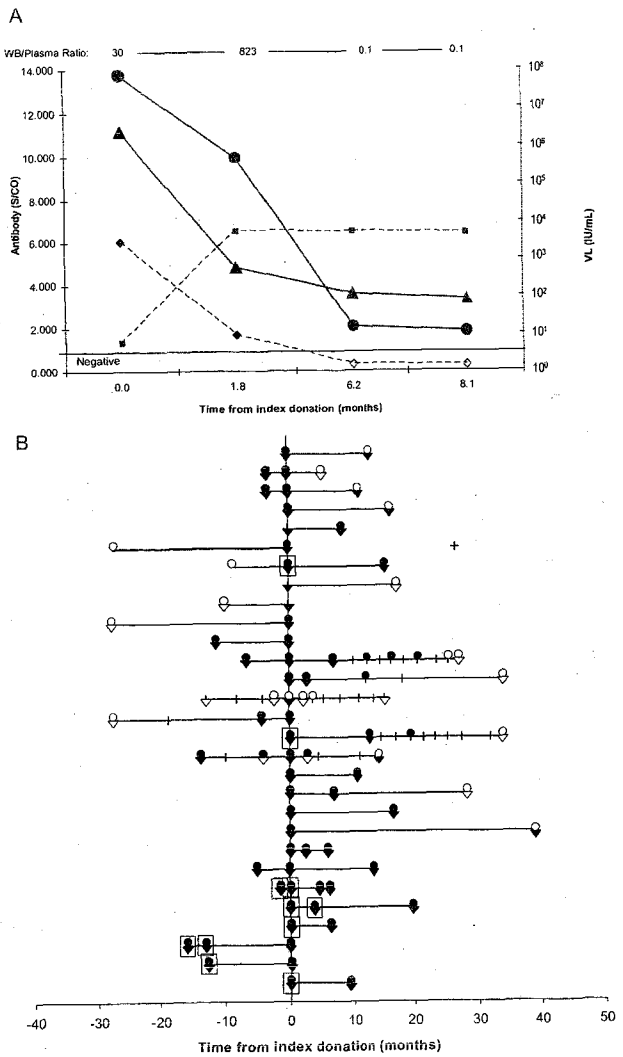
As part of our ongoing NAT research, we developed a new WB processing protocol using a novel buffer to precipitate and eliminate the Hb and other proteins which inhibit TC and PCR or transcription-mediated amplification. This unique buffer and associated protocol is termed "HemoBind" (patent pending) and delivers viral nucleic acids in a supernatant in a sufficiently pure form so that

TABLE 2. WB-to-plasma ratios for units with quantitative B19V DNA results of greater than 20 IU/mL

	WB-to-plasma DNA ratio					
	IgM positive			IgM negative		
Plasma DNA concentration	Number	Mean	Median	Number	Mean	Median
>20-100	6	4.43	2.15	18	2.19	1.06
>100	14	137.3	29.7	8	44.4	2.64
Total*	20	97.4	13.9	26	15.2	1.82

* Data for 58 IgM donations with DNA results that were negative or less than 20 IU/mL are not included.

Fig. 5. B19V distributions in blood compartments and persistence in infected RADAR donors. (A) B19V DNA concentration and serologic status over time for a blood donor with chronic persistent B19V infection. IgM (◆) and IgG (■) signal-to-cutoff (S/CO) ratio is plotted on the left *y*-axis, while the corresponding plasma (▲) and WB (●) B19V DNA concentrations in IU/mL are plotted on the right *y*-axis. The timing of the donation events is plotted on the *x*-axis. The WB-to-plasma ratio is indicated above the corresponding donation. (B) Quantitative B19V DNA results for all tested donations from 29 donors with at least one donation at a greater than 6-month interval. The *x*-axis represents time from index donation with the index donation plotted as Time 0. Additional donations going backward or forward from a positive index donation were tested by quantitative PCR, performed on both WB (●, ○) and plasma samples (▼, ▽). The closed symbols denote samples with detectable B19V DNA results and the open symbols denote negative B19V DNA results. IgM-positive donations are indicated by boxed symbols.



there is no inhibition of either the TC procedure or the subsequent amplification steps. Through spiking experiments, we demonstrated that combining HemoBind with TC and real-time PCR amplification yielded a quantitative B19V DNA assay that was efficient and reproducible in recovering B19V DNA spiked into WB over a wide spectrum of spiking concentrations.

We applied the HemoBind TC real-time PCR assay to paired WB and plasma samples from serial donations of RADAR donors who had been shown to have detectable plasma B19V DNA. During early stages of B19V infection when IgM was present and when B19V DNA concentrations were higher, we demonstrated that the median WB B19V DNA concentration was approximately 30 times higher than in plasma, and in some samples the WB-to-plasma ratio was more than 1000. In contrast, when IgM was absent later in infection and when B19V DNA concentrations were lower, the median WB-to-plasma ratio varied only by a factor of 1.06. Possible explanations for these results include the following: B19V is preferentially bound to RBCs when it is present in IgM immune complexes, more B19V is bound at higher plasma concentration due to steric effects on receptor-mediated binding, or B19V DNA is present at high levels within a subset of peripheral blood RBCs in the earlier compared to the later stages of B19V infection. This last hypothesis is intriguing given that B19V propagates in erythroblasts and the period of high-level cell-associated B19 viremia corresponds roughly to the 120-day survival period of RBCs in peripheral blood, and hence RBCs derived from infected erythroblasts could circulate for several months harboring B19V and account for the differential partitioning of DNA during the convalescent phase of infection. Further experiments that will be needed to investigate these possible explanations would require access to fresh blood specimens from acutely infected subjects to allow purification of intact cell subsets by centrifugation and elutriation or flow cytometry–based sorting, coupled with quantitative B19V detection analyses.

Although B19V infection had been classically considered to result in an acute transient infection, recent studies have established that chronic asymptomatic as well as symptomatic persistent B19V infections occur in a proportion of infected persons.[2-7] Such infections are characterized by prolonged periods with low levels of B19V DNA in blood of IgG-seropositive donors and patients. The rate of occurrence and determinants of persistent B19V infections are poorly understood. A few patients have been reported to have asymptomatic persistent B19 viremia for up to 3 to 5 years using highly sensitive PCR assays. Prolonged persistence of low-level plasma viremia has also been observed in follow-up studies of healthy donors.[2-7] In addition, recent studies indicate that B19V DNA can persist in solid tissues for years or even decades after clearance of circulating viremia and seroconversion.[5]

In this study, using a highly sensitive B19V DNA assay, we demonstrated that persistent B19V infection occurred in 88% of evaluable blood donors who had given at least one sample more than 6 months after plasma B19V DNA was initially detectable. B19V DNA levels either decreased from higher levels or remained at consistently low levels in these donors. These findings are similar to those in blood donor studies reported recently from Germany and Japan.[6,7] In addition, we found that B19V DNA persistence could also be detected by our WB assay, but without incremental detection relative to plasma; this is explained by the low titers of virus and absence of IgM during the persistent stage of infection.

While almost all donors demonstrated findings consistent with the known natural history of B19V infection, we did identify one donor with unusual results. B19V DNA was detectable at low levels in the absence of IgM and IgG antibody, which is contrary to the usual high viral loads (VLs; $>10^6$ IU/mL) that are detectable prior to antibody seroconversion. In addition, this donor did not seroconvert to IgG on a follow-up sample as would be expected. It is possible that this donor had a transient B19V infection (as indicated by the low DNA concentration) but for unknown reasons did not develop antibody to the VP2 antigen that is detectable by the antibody kits used in this study. We do not think the results can be explained by laboratory error since the positive index donation DNA results were obtained on both plasma and WB samples from this donation.

Since B19V is only rarely transmitted by blood transfusion, our finding of differential (high) WB-to-plasma concentrations in the early IgM-positive stages of infection have limited if any significance for blood safety. However, we believe that the general issue of differential levels of WB versus plasma nucleic acid concentrations for viruses and other blood-borne pathogens is important and encourage further development of sample preparative methods to enable performance of NAT on WB specimens. For transfusion-transmissible viruses with infectious window periods not currently detected by NAT screening, it is possible (though speculative) that WB nucleic acid testing could achieve greater sensitivity than plasma testing and thereby offer a tool to decrease residual risk. WB methods could also enable detection of persistent viral nucleic acids in convalescent or low-level persistent stages of infection (e.g., "occult HBV" and "elite controller HIV" infections), similar to our findings of more efficient detection of B19V during the convalescent IgM-positive phase of infection and the reported more efficient detection of WNV in WB relative to plasma in asymptomatic donors.[24] Also, adoption of high-throughput sample processing to allow use of WB in NAT assay systems would open the door to detection of cell-associated viruses and of parasitic and other nonviral agents, including those that are internal to RBCs (e.g., malaria or babesia para-

sites) or that partition with WBC fractions (e.g., *Trypanosoma cruzi*).

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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研究報告の概要

背景と目的：
我々は以前にパルボウイルス B19 遺伝子型 1 は血漿製剤由来の異なる溶液の液状加熱において異なる熱感受性パターンを示したことを報告した。本研究では、同様に B19 遺伝子型 2 を調べた。
材料と方法：
B19 の遺伝子型 1 と遺伝子型 2 の DNA を含む 2 つの血漿検体を用いた。アルブミン、免疫グロブリン、ハプトグロビンとアンチトロンビンの製造における熱処理工程の直前に採取した 4 つの工程検体に B19 をスパイクし、その後 60℃-10 時間の加熱処理をした。低 pH 免疫グロブリン溶液も B19 をスパイクし、室温で 14 日間の処理をし、感染性を測定した。
結　　果：
B19 遺伝子型 1 同様に、遺伝子型 2 は不活化の 3 つのパターンを示した。
(i)アルブミンと免疫グロブリンの急速な不活性化
(ii)ハプトグロビンの遅い不活性化
(iii)アンチトロンビンのみに限定した不活性化
低 pH 免疫グロブリン溶液中での感受性もまた、遺伝子型 1 に類似していた。
結　　論：
B19 の遺伝子型 1 と 2 の両方は、異なる血漿製剤の間で液状加熱と低 pH に対する感受性が変化した。

使用上の注意記載状況・その他参考事項等

代表としてテタノブリン IH 静注 250 単位の記載を示す。
1. 慎重投与
(1)略
(2)略
(3)溶血性・失血性貧血の患者〔ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。〕
(4)免疫不全患者・免疫抑制状態の患者〔ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、持続性の貧血を起こすことがある。〕
2. 重要な基本的注意
(1)略
1)血漿分画製剤の現在の製造工程では、ヒトパルボウイルス B19 等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。
6. 妊婦、産婦、授乳婦等への投与
妊婦又は妊娠している可能性のある婦人には、治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。〔妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルス B19 の感染の可能性を否定できない。感染した場合には胎児への障害（流産、胎児水腫、胎児死亡）が起こる可能性がある。〕

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報告企業の意見	今後の対応
万一、本剤の原料血漿にB19が混入したとしても、CPVをモデルウイルスとしたウイルスバリデーション試験成績及びB19を用いた不活化･除去試験の結果から、製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないものと考えるので、特段の措置はとらない。

抗破傷風人免疫グロブリン

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Variability of parvovirus B19 genotype 2 in plasma products with different compositions in the inactivation sensitivity by liquid-heating

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Vox Sanguinis

Background and Objectives Our previous report showed that parvovirus B19 genotype 1 in different solutions derived from plasma preparations showed different heat-sensitivity patterns during liquid-heating. In this study, we similarly examined B19 genotype 2.

Materials and Methods Two plasma samples one containing B19 genotype 1 and the other genotype 2 DNA were used. Four process samples collected immediately before the heat treatment step in the manufacture of albumin, immunoglobulin, haptoglobin and antithrombin preparations were spiked with B19 and subsequently treated at 60°C for 10 h. A low pH immunoglobulin solution was also spiked with B19 and treated at room temperature for 14 days. Infectivity was then measured.

Results B19 genotype 2, similar to genotype 1, showed three patterns of inactivation: (i) a rapid inactivation in the albumin and immunoglobulin preparations, (ii) a slow inactivation in the haptoglobin preparation and (iii) only limited inactivation in the antithrombin preparation. Its sensitivity in the low pH immunoglobulin solutions also resembled that of genotype 1.

Conclusion Both genotypes 1 and 2 of B19 varied in sensitivity to liquid-heating and low pH among different plasma preparations.

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Key words: genotype 2, heat & pH sensitivity, parvovirus B19, plasma products, viral inactivation.

Introduction

Human parvovirus B19 (B19) is not highly pathogenic to healthy individuals but can have serious effects in pregnant women and immunosuppressed individuals.

B19 has been long considered the only human pathogen in the genus *Erythrovirus* in the family *Parvoviridae* [1]. Its DNA sequence had been regarded as extremely stable, with variation of only approximately 1–2%. However, new variants (V9, A6 and Lali) have been reported to differ from genotype 1 by approximately 10% [2–4]. Genotypes 2 and 3 seem to have similar virulence to genotype 1 [5]. Servant *et al.* [6] proposed classifying human erythroviruses into genotypes 1 (prototype Au), 2 (Lali-like) and 3 (V9-like). Genotype 1, the major form of B19, has spread worldwide. Genotype 2 has been reported mostly in Europe and North America. At present, only small numbers of donor plasma samples are believed to contain high levels of genotype 2 [7]. In genotype 1, the structural proteins of the capsid consist of approximately 96% VP2 (58 kDa) and 4% VP1 (84 kDa). VP1 differs from VP2 only in a unique N-terminal region (VP1u), where it has an additional 227 amino acids

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[8]. However, there have been no reports on morphological structure and protein composition in genotype 2.

B19 genotype 1 DNA has been detected by PCR in plasma products, such as coagulation factors [9, 10]. Genotype 2 DNA has also been detected in 2·5% of coagulation factor concentrates [11]. Despite measures taken against viral contamination, including improved testing and inactivation and/or removal steps during the manufacturing process, the potential for B19 transmission through plasma products remains. B19 genotype 1 has been shown to be sensitive to liquid-heating than other animal parvoviruses such as canine parvovirus (CPV) and porcine parvovirus (PPV) [12–15]. We have also shown that B19 genotype 1 in different solutions showed different heat-sensitivity patterns during liquid-heating: (i) a rapid inactivation in *albumin or immunoglobulin preparations, (ii) a slow inacti*vation in haptoglobin preparations and (iii) only limited inactivation in antithrombin preparations [14]. Genotype 2 was also shown to be rapidly inactivated in 5% albumin at 56°C [16]. The effective inactivation of both genotypes in coagulation factors, which contained 7–8% residual moisture, during vapour-heating (STIM-4) at 60°C for 500 min followed by at 80°C for 60 min has also been reported [17]. In the case of freeze-dried products, the sensitivity of B19 genotype 1 to heat treatment has been shown to be influenced by the residual moisture in the sample [18]. Therefore, B19 genotype 2 seems to be effectively inactivated under high residual moisture and high temperature conditions. However, it remains unknown whether the heat sensitivity of genotype 2 varies in different types of products.

In this study, we examined the inactivation kinetics of genotype 2 during liquid-heating and low pH treatment in several manufacturing processes using a recent B19 isolate derived from rejected donor plasma containing B19 genotype 2. In addition, the full nucleic acid sequence, the morphological structure, and the protein composition of the genotype 2 isolate also investigated.

Materials and methods

Viruses

Two plasma samples containing either B19 genotype 1 or genotype 2 DNA, collected from donors in the USA and named F15 and F27, respectively, were used. Each sample was converted to serum by the addition of calcium chloride. The amount of genome in F15 and F27 was quantified as 11·2 and 11·0 Log_{10} IU/ml, respectively (Artus™ Parvo B19 TM PCR kit; Artus GmbH, Hamburg, Germany). The samples were also examined for viral markers as described below, according to good manufacturing practices. They tested negative for anti-human immunodeficiency virus (HIV)-1/2 IgG, hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus (HCV) IgG and anti-B19 IgG in immunoassays. In addition, HIV RNA, hepatitis A virus RNA, HBV DNA and HCV RNA were not detected using nucleic acid amplification tests.

Test materials

In this study, we used a 25% albumin preparation containing sodium caprylic acid/acetyl tryptophan, a haptoglobin preparation containing glycine, an antithrombin preparation containing sodium chloride/tri-sodium citrate dihydrate and an IVIG preparation containing D-sorbitol, respectively, as stabilizers. To mimic the manufacturing process conditions, test samples were collected immediately before the heat treatment of the albumin, haptoglobin and antithrombin preparations (Albumin25%$_{I.V.-BENESIS}$, Haptoglobin$_{I.V.}$2000$_{units-BENESIS}$ and Neuart®$_{I.V.}$, respectively; Benesis Corporation, Osaka, Japan). To avoid interference with the infectivity assay by neutralizing antibody against B19 during heat or low pH treatments, an immunoglobulin sample derived from anti-B19 IgG-negative plasma was prepared under the same manufacturing conditions as Venoglobulin®IH5%$_{I.V.}$ (Benesis Corporation). The samples were collected immediately before the heat or low pH treatment.

For neutralization assays, a commercial product, intravenous immunoglobulin (IVIG, Venoglobulin®IH5%$_{I.V.}$ including anti-B19 IgG), was used.

Inactivation and neutralization of viruses

The process samples described above were investigated by liquid-heating as described previously [13–15]. The samples spiked with B19 were divided into aliquots and heat treated in water bath at 60°C for preset periods. The collected samples were immediately cooled to make a 10-fold serial dilution and incubated with the KU812 cells. The infectivity remaining in each sample was measured by the subsequent detection of spliced B19 mRNA in cultured cells, as described [13, 14]. Two independent inactivation experiments were performed. The viral titre (log_{10}) in one experiment was determined by *non-detectable end-point* dilution [13] and in the other experiment by tissue culture infectivity dose 50% ($TCID_{50}$) according to the Kärber method [14], respectively.

The forward primers and the reverse primers were defined as follows. The primer set of B19-25 (nt 1897–1916; 5′-GTCGGAAGCCCAGTTTCCTC-3′ as 5′ sense) and B19-11 (nt 2962–2943; 5′-TGCACCAGTGCTGGCTTCTG-3′ as 3′ antisense) was used for the non-detectable end-point dilution method, and another primer set of B19-21 (nt 1961–1980; 5′-TGGCAGACCAGTTTCGTGAA-3′ as 5′ sense)

and B19-22 (nt 2886–2866; 5′-CCGGCAAACTTCCTTGAAAA-3′ as 3′ antisense) was used for $TCID_{50}$.

Both viruses were also tested for inactivation at low pH. The viruses were incubated in IVIG sample (pH 4·15), IVIG buffer without IgG (5% Sorbitol, pH 4·15) or cell culture medium at room temperature for a preset period. The low pH reaction was stopped by addition of 1:9 volumes of culture medium. Run control studies comprising the same experimental conditions without the virus inactivation step were also performed in each study as a control. Cytotoxicity tests were also performed, and infectivity was then measured in the non-cytotoxic range as described above.

Neutralization titres of IVIG samples against B19 viruses were measured as previously reported [15].

Cloning and sequencing of the B19 genotype 2 genome

Restriction enzymes, the DNA polymerase I Klenow fragment and a DNA Ligation kit, Ver. 2 (Takara Bio Inc., Shiga, Japan), were used for cloning. Viral DNA was extracted from F27 without amplification using Smitest Ex-R&D (GenomeScience Laboratories Co., Fukushima, Japan). The DNA was cleaved by cutting inverted terminal repeats (ITRs) in half with *BssH*II, treated with the Klenow fragment to fill the 3′-end, and ligated into the *Hind*II site of pUC19. The ligated DNA was transfected into competent DH5α cells (Toyobo Co. Ltd., Osaka, Japan). Each clone was sequenced using a 3130*xl* genetic analyzer (Applied Biosystems Japan Ltd, Tokyo, Japan) and BigDye Terminator Cycle Sequencing kit (Applied Biosystems Japan Ltd). Full-length sequences were obtained by primer walking. The entire sequence of F27 has been deposited in the DDBJ/GenBank/EMBL nucleotide databases (accession number AB550331).

Electron microscopy and sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE)

Each plasma sample was diluted threefold with phosphate-buffered saline (PBS) and then ultracentrifuged at 154 000 *g* for 3 h. The precipitate was resuspended with PBS, and the viral fraction in the suspension was purified by caesium chloride density gradient ultracentrifugation at 210 000 *g* for 26 h. The fractions were collected, and the amount of B19 DNA in each was quantified using an Artus™ Parvo B19 TM PCR kit (Artus GmbH).

For transmission electron microscopy (TEM), the B19 DNA-rich fractions were dialysed in 50 μM ammonium acetate overnight and 8% paraformaldehyde in 50 μM ammonium acetate added to a final concentration of 2% to inactivate the virus. Specimens for negative staining were made as follows; inactivated samples were applied to carbon-coated electron microscope grids (200-A copper grid; Nisshin EM Co., Tokyo, Japan) for 5 min, and then the excess was removed by blotting with filter paper. Once on the carbon-coated grids, samples were stained with 2% (w/v) uranyl acetate for 30 s, blotting with filter paper and dried in air. Negatively stained specimens were observed by TEM at 100 kV (JEM-100S; JEOL Datum Ltd., Tokyo, Japan). Whole micrographs were digitized as 8-bit images using a scanner (GF ×800; Seiko EPSON Co., Nagano, Japan) and then analysed with the software EMAN (version 1.8, http://blake.bcm.tmc.edu/eman/eman1/) and ImageJ (version 1.36b, http://rsb.info.nih.gov/ij/index.html).

The B19 DNA-rich fractions were precipitated with 10% (w/v) trichloroacetic acid, and the precipitates were solubilized in a buffer containing 2% SDS, 5% (v/v) 2-mercaptoethanol, 0·01% (w/v) bromophenol blue and 20% (w/v) glycerol for 3 min at 100°C. Aliquots were electrophoresed in 10% polyacrylamide gel (Bio-Rad Laboratories, K. K., Tokyo, Japan) and subjected to Coomassie brilliant blue (CBB) staining.

Results

Inactivation of B19 genotypes 1 and 2 during liquid-heating

The infectivity of B19 genotype 2 (F27 isolate) in each sample was measured by detecting B19 spliced mRNA in infected KU812 cells. The B19 genotype 2 virus was rapidly inactivated at 60°C in the 25% albumin and immunoglobulin preparations by at least 4·6 logs (Fig. 1a, b). It was slowly inactivated in the haptoglobin preparation (Fig. 1c). The virus was only partially inactivated, i.e. 2·6 logs in the antithrombin preparation even after 10 h at 60°C (Fig. 1d). The viruses showed retained infectivity in the run control study (data not shown). Interestingly, the inactivation kinetics of genotypes 1 and 2 in each product were almost the same.

Inactivation of B19 genotypes 1 and 2 at low pH

The B19 virus samples were also incubated in the immunoglobulin preparation (pH 4·15). Both genotype 1 and 2 viruses were inactivated below detectable limits, i.e. by >3 logs at day 1 (Fig. 2). Both B19 viruses in the IVIG sorbitol buffer, used as a control, were also inactivated beyond detection at day 7. B19 infectivity was retained in the run control study (data not shown). The kinetics demonstrated that genotypes 1 and 2 showed similar sensitivity to low pH treatment.

Neutralizing activity of IVIG against genotype 2

IVIG showed neutralizing activity against genotype 2 as well as genotype 1 (Table 1). The result suggested that anti-B19 genotypes 1 and 2 neutralizing IgG was contained in the IVIG products.

Cloning and sequencing of the genome of isolate F27

The viral genome of F27 was cloned. The nucleotide and amino acid sequences were compared with those of strains Au (genotype 1; GenBank accession No. M13178), A6 (genotype 2; GenBank accession No. AY064475) and V9 (genotype 3; GenBank accession No. AX003421). The homology between F27 and the Au, A6 and V9 strains was 87·7%, 98·1% and 91·1%, respectively. The amino acid sequences of VP1 and VP2 were highly conserved (97·4% and 98·6%) between F27 and Au.

It has been reported that four basic regions in VP1u exist among animal parvoviruses [CPV, PPV and minute virus of mice (MVM)] [19]. The overall percentage of basic amino

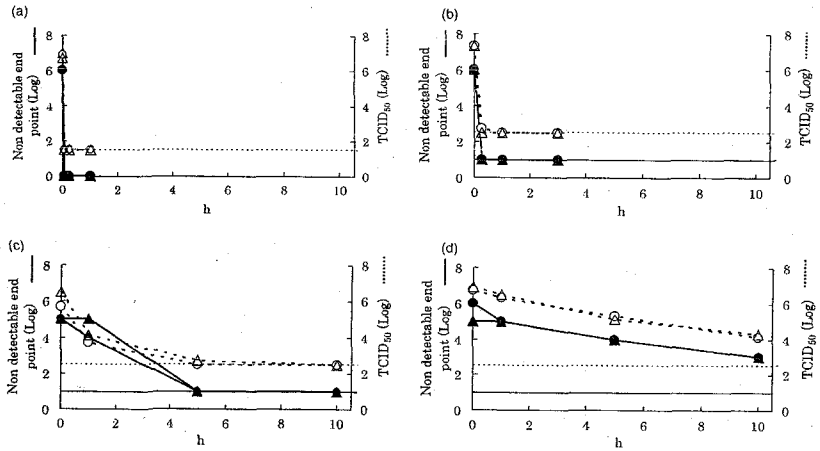

Fig. 1 The kinetics of heat-induced inactivation of B19 genotypes during the manufacturing process. Samples spiked with B19 were collected immediately before and after heat treatment at 60°C using 25% albumin (a), immunoglobulin prepared from anti-B19 immunoglobulin-negative plasma (b), haptoglobin (c) and antithrombin (d) preparations. The infectivity remaining in each sample of B19 was measured by non-detectable end-point dilution [genotype 1 (●) and genotype 2 (▲), solid lines] and by $TCID_{50}$ [genotype 1 (○) and genotype 2 (△), dashed lines], respectively, as described in Materials and Methods. The solid and dotted horizontal lines represent the detection limits of the assays.

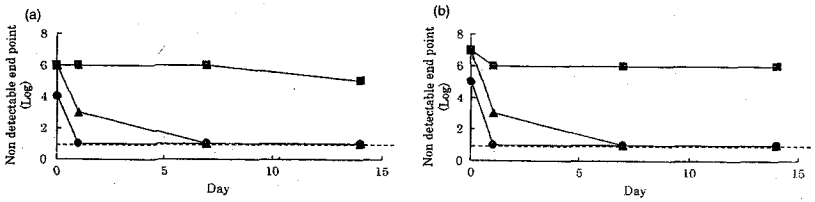

Fig. 2 The kinetics of low pH-induced inactivation of B19 genotype 1 strain F15 (a) and genotype 2 strain F27 (b) during the IVIG manufacturing process. The infectivity remaining in each sample of the immunoglobulin preparation (pH 4·15) derived from anti-B19 IgG-negative plasma (●), 5% sorbitol buffer control (pH4·15; ▲) and culture medium pH7 (■) was measured by non-detectable end-point dilution with two independent experiments. The dashed horizontal lines represent the detection limit.

Table 1 Neutralization of B19 genotypes with intravenous immunoglobulin

Dilution (fold)	Total IgG/reaction (μg)	Total antibody titre (IU)	Genotype 1 DNA (9·7 log copies)	Genotype 2 DNA (9·7 log copies)
1	2500	13·90	–	–
3	830	4·63	–	–
9	280	1·54	–	+
27	90	0·51	+	+
81	30	0·17	+	+

+, infectious to KU812 cells; –, non-infectious to KU812 cells.
Total reaction volume was 0·6 ml.

acids (arginine and lysine) in the viruses including F27 is summarized in Table 2. Interestingly, 8·8% of basic amino acids with no basic region in VP1u of F27 and 8·8% of basic amino acids with only one basic region in Au were also confirmed.

Electron microscopic observation and protein composition of viral particles

Native particles of genotype 2 were first observed by TEM (Fig. 3). The genotype 1 and 2 particles were of similar shape: mostly hexagonal and sometimes pentagonal. Mean diameters were measured at the shorter axis and were 22·0 ± 1·9 nm (n = 155) and 22·5 ± 1·5 nm (n = 242), respectively.

Native particles were also subjected to SDS–PAGE in a 10% gel, followed by staining with CBB (Fig. 4). Both genotype 1 and 2 particles also comprised the major 58-kDa protein VP2 and minor 84-kDa protein VP1. Western blotting using the anti-B19 polyclonal antibody confirmed that these proteins are viral structural components (data not shown).

Table 2 The numbers of basic amino acids and basic regions within the VP region of three animal parvoviruses and parovirus B19 genotypes 1 and 2

	Basic amino acids/total amino acids		Number of basic regions	
	VP1u	VP2	VP1u	VP2
CPV	29/167 (17·4)	43/584 (7·4)	4	2
PPV	29/170 (17·1)	38/579 (6·6)	4	0
MVM	24/142 (16·9)	44/587 (7·5)	4	1
B19 genotype 1	20/227 (8·8)	42/554 (7·6)	1	0
B19 genotype 2	20/227 (8·8)	42/554 (7·6)	0	0

The GenBank accession numbers of the five isolates are M19296 (CPV; strain CPV-N), D00623 (PPV; strain NADL-2), J02275 (MVM; strain MVM(pI), M13178 (B19 genotype 1 strain Au) and AB550331 (B19 genotype 2 isolate F27).
The values in parentheses are shown as percentages.
CPV, canine parvovirus; MVM, minute virus of mice; PPV, porcine parvovirus.

Discussion

In this study, we have shown that the heat-sensitivity patterns of B19 genotype 2 varied in different solutions during liquid-heating: (i) rapid inactivation in albumin and immunoglobulin preparations, (ii) slow inactivation in haptoglobin preparations and (iii) only limited inactivation in antithrombin preparations. The results revealed that in terms of sensitivity to heat, B19 genotype 1 and 2 viruses are similar to each other. The stabilizers in plasma preparations are selected based on the ability to stabilize plasma proteins. The composition of the test sample is an important factor in the evaluation. Thus, to mimic manufacturing conditions, samples were collected just prior to the heating process.

Only limited B19 inactivation occurs in some products during liquid-heating. Therefore, to further improve the safety of plasma products, different or additional methods of inactivation and/or removal are desirable [20]. In our albumin, IVIG, haptoglobin and antithrombin preparations, robustness was ensured by employing steps other than heating. A 15-nm filter is used for the antithrombin preparation. Observations by TEM showed the mean diameter of B19 genotype 1 and 2 particles to be 22·0 and 22·5 nm, respectively. Previous studies have shown that filtration of a haemoglobin solution resulted in a more than 6-log reduction in B19 genotype 1 [21]. Given the similarity in size, the 15-nm filter would be effective for the removal of genotype 2 as well as genotype 1 particles. B19 genotype 1 also showed heat resistance properties in sodium chloride/tri-sodium citrate dehydrate buffer whereas the virus was inactivated rapidly in sodium chloride or tri-sodium citrate dehydrate buffer alone (data not shown). The results suggested that the heat sensitivity of the B19 is affected by a number of factors and appears to be complex. Thus, B19 heat sensitivity during heat treatment should be evaluated for in each process condition to avoid an over estimate of the level of B19 inactivation.

Recently, information on the mechanisms by which viruses are inactivated during liquid-heating has been obtained using B19 and MVM. The genomes of B19 and MVM were released with increasing temperature and subsequently only empty capsids remained. Interestingly, B19 was released at a lower temperature than MVM. This phenomenon seemed to be related to the heat inactivation kinetics of B19 and MVM [22, 23]. In addition, the VP1u region of B19 or MVM seemed to remain inaccessible in the capsid before heat treatment, because an anti-B19 VP1u antibody could not bind to viral particles, whereas it could after heating. It was suggested that VP1u became exposed on the surface of particles during heating [24, 25]. VP1u may be involved in movement through the pores of the nuclear membrane in animal parvoviruses, because it is rich in basic amino acids (arginine and lysine), which is typical of many DNA-binding proteins [19]. The percentage of basic amino acids in the major capsid protein VP2 of CPV, PPV and MVM was 6·6–7·5%. That in VP1u was around 17%.

Although absolute molecular sizes were not clarified, we confirmed that VP1 and VP2 of B19 genotype 2 were similar in size to genotype 1 by SDS–PAGE of viral particles. Interestingly, the percentages of basic amino acids in VP1u between B19 genotypes and animal parvoviruses were significantly different. The basic region in VP1u and binding with viral DNA in animal parvoviruses may be involved in the stabilization during heat inactivation. Therefore, the different properties of the binding of VP1u with viral DNA may influence the heat sensitivity of B19.

The inactivation kinetics of genotype 2 at low pH was almost the same as that of genotype 1. Blümel *et al.* [16] also reported similar properties. The inactivation mechanism at low pH was also considered, to be similar to that for heat treatment [21–23].

The neutralizing activity of IVIG against genotypes 1 and 2 was similar. This is because of the presence of a wide range of neutralizing antibodies in IVIG derived from over 10 000 healthy donors [26]. When a renal transplant recipient infected with B19 genotype 2 was treated with high-dose IVIG, the viral load in serum was reduced and the symptoms improved [27]. This clinical study also suggests that IVIG contains neutralizing activity towards the genotype 2 virus.

In conclusion, the inactivation kinetics on liquid-heating depends on the composition of the solution, and low pH treatment of native B19 genotype 2 virus had a remarkably similar effect to that on genotype 1. Although B19 is classified in the family *parvoviridae*, it differs from other animal parvoviruses in that it is more variable to inactivation. Further study is needed to clarify the mechanism behind the heat resistance of B19.

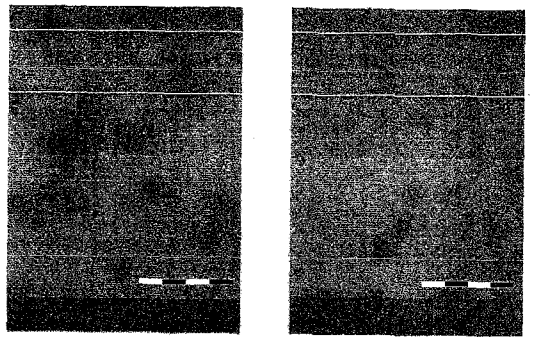

Fig. 3 Electron micrographs of purified native B19 particles. Field view of genotype 1 strain F15 (left) and genotype 2 strain F27 (right). The bar represents 400 nm. Inlet shows isolated particles. Each rectangle is 108 nm wide and 87 nm high.

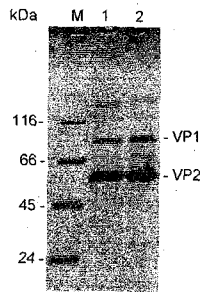

Fig. 4 Coomassie brilliant blue staining of the structural proteins of native particles of B19 genotypes 1 and 2. Purified viral particles were reduced and subjected to SDS–PAGE. Lanes M, 1 and 2 show the molecular marker, genotype 1 strain F15 and genotype 2 strain F27, respectively.

Acknowledgements

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一般的名称	①②ポリエチレングリコール処理抗破傷風人免疫グロブリン ③乾燥抗破傷風人免疫グロブリン		研究報告の公表状況	Journal of Virological Methods 2011;178(1-2):39-43	公表国 日本	
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-80-

研究報告の概要

製造工程の異なる段階での生物学的製剤の検査は、現在では定量ポリメラーゼ連鎖反応(Q-PCR)ベースの分析を含む。Q-PCR 技術は、ウイルス粒子内のウイルスゲノムだけでなく、検体中の分解したゲノムの断片をも検出可能である。15～19nm フィルターのウイルス除去能について、最小のノン・エンベロープウイルスの一つであるパルボウイルス B19(B19)を用いて、感染力分析と Q-PCR 分析を行うことにより調査した。フィルター処理された検体は感染力を示さなかったが、ウイルス DNA は Q-PCR によって検出された。
興味深いことに、15nm 濾液中の全ウイルスゲノムの約 90%は、Q-PCR により 0.5kb 未満の検出可能サイズを有していた、結果としてリダクションファクターは Q-PCR を用いて過小評価されていた。
Q-PCR を用いたリダクションファクターは、遊離 B19 DNA の大量の存在により過小評価されるかもしれない。
従って、Q-PCR の結果は慎重に解釈されるべきである。Q-PCR によるウイルス DNA の断片から増幅を排除するためには、プライマーの慎重な設計が必要である。

使用上の注意記載状況・
その他参考事項等

代表としてテタノブリン IH 静注 250 単位の記載を示す。
1. 慎重投与
(1)略
(2)略
(3)溶血性・失血性貧血の患者〔ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。〕
(4)免疫不全患者・免疫抑制状態の患者〔ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、持続性の貧血を起こすことがある。〕
2. 重要な基本的注意
(1)略
1)血漿分画製剤の現在の製造工程では、ヒトパルボウイルス B19 等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。
6. 妊婦、産婦、授乳婦等への投与
妊婦又は妊娠している可能性のある婦人には、治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。〔妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルス B19 の感染の可能性を否定できない。感染した場合には胎

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報告企業の意見	今後の対応	児への障害（流産、胎児水腫、胎児死亡）が起こる可能性がある。）
ヒトパルボウイルスB19（human parvovirus B19：B19）は、脂質エンベロープを持たない極めて小さな（約20～26nm）DNAウイルスで、輸血や血漿分画製剤による伝播が報告されている。他のウイルスに比べて、血漿分画製剤の製造工程での不活化・除去が困難であり、本ウイルスの伝播リスクを完全に否定することはできないため、1996年11月より、使用上の注意にB19についての記載を行い注意喚起を図ってきた。万一、原料血漿にB19が混入したとしても、CPVをモデルウイルスとしたウイルスクリアランス試験成績及びB19を用いた不活化・除去試験の結果から、本剤の製造工程において十分に不活化・除去されると考えている。なお、原料血漿へのB19混入量低減のため、B19-ミニプール-NATが米国の原料供給元で行われている。	本報告は本剤の安全性に影響を与えないものと考えるので、特段の措置はとらない。	

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Caution in evaluation of removal of virus by filtration: Misinterpretation due to detection of viral genome fragments by PCR

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ABSTRACT

The testing of biological products at different stages of the manufacturing process currently involves quantitative polymerase chain reaction (Q-PCR)-based assays. Q-PCR techniques are able to detect not only the viral genome in viral particles but also fragments of degraded genome in samples. The ability of 15 and 19-nm filters to remove viruses was examined by conducting infectivity assays and Q-PCR assays using parvovirus B19 (B19), one of the smallest non-enveloped viruses. Although the filtered samples showed no infectivity, viral DNA was detected by Q-PCR. Interestingly, approximately 90% of the total viral genome in 15-nm filtrates had a detectable size of less than 0.5 kb by the Q-PCR and as a consequence reduction factors were underestimated using Q-PCR. The reduction factors using Q-PCR might be underestimated due to the presence of a large amount of free B19 DNA which shows no *infectivity in the tested filtrates. Therefore, the results of Q-PCR should be interpreted with caution.* The careful design of primers is needed to eliminate amplification from fragments of viral DNA by Q-PCR.

1. Introduction

The implementation of polymerase chain reaction (PCR)-based testing for viral genomes in donated plasma (usually in mini-pools) has contributed to reductions in viral load during the manufacture of plasma pools (Roth et al., 2000). Heat treatment which inactivates many classes of virus as well as solvent/detergent treatment which only inactivates enveloped viruses, and also several steps in the manufacture of plasma products have helped to reduce the risk of potential transmission of viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). Filtration using membranes with a pore size of only a few nanometers (nm), often called nano-filtration, is one of the most effective means of removing non-enveloped viruses such as parvovirus B19 (B19) and hepatitis A virus (HAV), as well as enveloped viruses. Nanofiltration has been applied to the manufacturing of various plasma products (Burnouf and Radosevich, 2000, 2003). Generally, infectivity assays have been implemented for the quantitation of viruses in samples according to guidelines for virus clearance studies (ICH, 1997; EMEA, 1996; MHLW Japan, 1999). Recently, quantitative PCR (Q-PCR) techniques which demonstrate a high level of precision and fast throughput have been introduced to evaluate the removal of viruses through filtration (Lovatt, 2002).

In this study, infectivity and Q-PCR assays were compared for the evaluation of virus removal over 15 and 19 nm filters using B19 as model virus. *The study revealed the presence of B19 in samples* that were found positive by Q-PCR but which was not infectious, implying that this Q-PCR signal could be derived from DNA fragments of the viral genome. *Therefore, the data obtained by Q-PCR* should be interpreted with caution.

2. Materials and methods

2.1. Viruses

A B19 genotype 1-positive plasma sample (termed F15) was used in these studies. The plasma was collected by a licensed establishment in the USA according to local regulations. The number of genomes in F15 was quantified as 11.2 Log_{10} International Unit (IU) (Saldanha et al., 2002)/mL (Artus™ Parvo B19 TM PCR Kit, Artus, Hamburg, Germany). F15 was also tested for other viral markers,

Abbreviations: B19, parvovirus B19; BMM, Bemberg Microporous Membrane; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; *IVIG, intravenous immunoglobulin; LRF, Log_{10} reduction* factor; PCR ELISA, polymerase chain reaction enzyme-linked immunosorbent assay; Q-PCR, quantitative polymerase chain reaction.

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according to good manufacturing practices: it tested negative for anti-HIV-1/2 IgG, HBV surface antigen (HBsAg), anti-HCV IgG and anti-B19 IgG in enzyme immunoassays. In addition, HIV-RNA, HAV-RNA, HBV-DNA and HCV-RNA were below the limit of detection using nucleic acid amplification tests. All plasma samples were converted to serum by the addition of a 1:20 volume of 4% $CaCl_2$ followed by incubation at 32 °C for 30 min. Fibrin clots were removed with a Büchner funnel and the samples stored at −80 °C.

2.2. Test materials

Samples collected immediately before the nano-filtration steps in the manufacturing process of antithrombin, haptoglobin and intravenous immunoglobulin (IVIG) products (Neuart®$_{I.V.}$, Haptoglobin$_{I.V.}$ and Venoglobulin®IH 5%$_{I.V.}$, respectively; Benesis Corp., Osaka, Japan) were used. These products have been manufactured using source plasma derived from healthy volunteer donors in Japan. The plasma pools used for manufacture of the products was also tested for the viral markers listed above and all were negative. In addition, B19 virus antigen was screened using a receptor-mediated hemagglutination assay (Sakata et al., 1999).

2.3. Removal of viruses by filters

B19 was purified by ultracentrifugation at 150,000 × *g* for 3 h and the resultant pellet was re-suspended in respective start material for each filtration experiment. The suspensions were sonicated to disperse the virus and passed through a 0.22-μm filter (SLGV033RS, Millipore Corp., Billeria, MA). For IVIG solutions, to ensure the formation of virus–antibody complexed forms, B19 was added to IVIG samples and adjusted to pH 5.5 where a complex of anti-B19 antibody in IVIG with B19 particles forms. The sample was sonicated, passed through a 0.22-μm filter, made up to required volumes for filtration, and adjusted to pH 4.2. The B19-spiked antithrombin sample was subjected to filtration using Planova-15N (Asahi Kasei Medical Corp., Tokyo, Japan, 15 ± 2 nm, 0.001 m^2 module). The B19-spiked haptoglobin and IVIG samples were subjected to filtration using Planova-20N (Asahi Kasei Medical, 19 ± 2 nm, 0.001 m^2 module). Conditions for the filtration mimicked (i.e. appropriately down-scaled) the manufacturing conditions for each product according to published guidelines (ICH, 1997; EMEA, 1996; MHLW Japan, 1999). The filtration of each preparation was performed in two runs.

2.4. Infectivity assay of B19

The infectivity assay for B19 utilised cell culture followed by a PCR detection of spliced mRNA as described previously (Hattori et al., 2007). The infectivity of B19 in the samples was titered using KU812 cells (JCRB0104; Health Science Research Resources Bank, Tokyo, Japan) and subsequently viral mRNA expressed in infected wells was detected by RT-PCR. Briefly, 5.3 Log_{10} cells were inoculated with 0.1 mL of 10-fold serial dilutions of samples and cultured in RPMI-1640 medium containing 10% fetal bovine serum, 6 IU/mL of erythropoietin (Sankyo Co., Ltd., Tokyo, Japan), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Invitrogen Corp., Carlsbad, USA), 1 mL of sodium pyruvate (Invitrogen Corp.), and an ITS-X supplement (Invitrogen Corp.) for 4 days. Wells were monitored for the presence or absence of viral mRNA. The spliced viral mRNA was detected by RT-PCR and agarose gel electrophoresis followed by staining with ethidium bromide. The sense primer was B19-25 (nt 2098–2117; 5′-GTCGGAAGCCCAGTTTCCTC-3′) and the antisense primer was B19-11 (nt 3163–3144; 5′-TGCACCAGTGCTGGCTTCTG-3′), where the nt numbers refer to the Au sequence (GenBank Accession No. M13178). The RT-PCR titre (Log_{10} dilution) was determined by end-point dilution and the result was considered positive when the amount of B19 genome determined with the Artus Kit was more than 5.3 Log_{10} IU/mL (4.3 Log_{10} IU/well). Control runs, with the same experimental conditions but without filtration, were also performed. Cytotoxicity tests were also performed and infectivity was measured in the non-cytotoxic range as described above.

2.5. B19 DNA assays

B19 DNA in samples before and after filtration was extracted with a QIAamp viral RNA mini kit (QIAGEN Inc., Valencia, CA) and quantified using a quantitative PCR assay kit (Artus™ Parvo B19 TM PCR Kit). The kit was used in accordance with the manufacturer's instructions using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster, CA). Briefly, the reaction mixture that consisted of 30 μL of Master Mix and 10 μL of purified DNA was brought up to a final volume of 50 μL with distilled water. The reaction profile was as follows: 95 °C for 10 min, then 45 cycles of 95 °C for 15 s and 60 °C for 1 min. A 76-bp region of the B19 genome was amplified and the product was detected by measuring the fluorescence of FAM-labeled probe which hybridized specifically to the amplicon. For the quantitative assay, a full set of five Quantitation Standards served as positive controls and results were expressed in international units (IU) (Saldanha et al., 2002) per milliliter. The range of linearity was from 10 IU to 6 Log_{10} IU/reaction and the detection limit was 4 IU/reaction.

PCR ELISA is a qualitative method of detecting B19 DNA by labeling amplicons with digoxigenin (DIG) (Hattori et al., 2007). The samples were diluted 10-fold and B19 DNA was extracted from the samples using Smitest Ex R&D (Genome Science Laboratories, Aichi, Japan), following the manufacturer's instructions. Five volumes of protease solution were added to 1 volume of sample and incubated at 55 °C for 30 min. After proteolysis, 4 volumes of protein denaturation solution were added, incubation continued at 55 °C for 15 min, and then 8 volumes of isopropanol were added, and the mixture was placed on ice for more than 15 min. After centrifugation at 20,000 × *g* for 20 min, pellets were washed with 70% ethanol twice. The region encoding the B19 V region (376 bp) was amplified using a PCR ELISA (DIG-Labeling) Kit (Roche Diagnostics, Mannheim, Germany) with primers B1 (nt 3187–3206; 5′-CAAAAGCATGTGGAGTGTGAGG-3′) and B2 (nt 3558–3539; 5′-GTGCTGTCAGTAACCTGTAC-3′). The amplicon incorporates the DIG-labeled nucleotide during the PCR. The DIG-labeled PCR product was detected using a PCR ELISA (DIG-Detection) Kit (Roche Diagnostics). The amplicon was denatured and hybridized with a biotin-labeled oligonucleotide, probe B (nt 3310–3339; 5′-TAGCTGCCACAATGCCAGTGGAAAGGAGGC-3′), in which nt numbers refer to the Au sequence (GenBank Accession No. M13178). This hybrid was immobilized on a streptavidin-coated microplate and detected with a peroxidase-conjugated anti-digoxigenin antibody and the colorimetric substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The PCR titre was determined by end-point dilution.

2.6. Agarose gel electrophoresis and recovery of DNA from gel

B19 DNA was extracted using Smitest Ex R&D (see above). The pellets were dissolved in 10 μL of 50 mM Tris–HCl (pH 7.5) buffer containing 100 mM NaCl and anealed at 60 °C for 30 min. One microgram of a 1-kb DNA Ladder (Invitrogen, Carlsbad, CA) was added to the extract and an aliquot was subjected to gel electrophoresis with 1.0% SeaKem GTG agarose (Lonza, Rockland, ME). The agarose gel was stained with ethidium bromide and cut into five sections (<0.5, 0.5–1.0, 1.0–2.0, 2.0–4.0 and >4.0 kb). DNA was extracted from each section using a QIAquick Gel Extraction Kit (QIAGEN) in accordance with the manufacturer's instructions.

Table 1
LRF of B19 infectivity and Q-PCR signals by three filtration processes.

Preparations pore size	Antithrombin 15 nm		Haptoglobin 19 nm		IVIG 19 nm	
Method	Infectivity (NDP)	Q-PCR (Log_{10} IU/mL)	Infectivity (NDP)	Q-PCR (Log_{10} IU/mL)	Infectivity (NDP)	Q-PCR (Log_{10} IU/mL)
Pre	5.0/6.0	9.8/9.8	4.0/4.0	8.7/8.7	<1.0/<1.0	8.8/8.4
Post	<1.0/<1.0	5.0/5.3	<1.0/<1.0	5.3/5.4	<1.0/<1.0	6.3/6.2
LRF	≥4.0/≥5.0	4.8/4.5	≥3.0/≥3.0	3.4/3.3	*/*	2.5/2.2

LRF, log reduction factor; NDP, non-detectable end-point; *, could not be calculated.

Briefly, three volumes of Buffer QG were added to 1 volume of gel and incubated at 50 °C for 10 min. After the gel slice was dissolved completely, one volume of isopropanol was added, and the mixture was placed in a spin column and centrifuged at 17,900 × *g* for 1 min. Next, 0.75 mL of washing Buffer PE was added, and the spin column was centrifuged at 17,900 × *g* for 1 min and then centrifuged once more. To elute DNA, 50 μL of water was placed on the column and centrifuged at 17,900 × *g* for 1 min. The B19 DNA in the extract of each section was quantified with the Artus B19 Kit.

3. Results

3.1. Removal of B19 in three steps

The removal of B19 was examined using both infectivity assays and the amount of viral genome determined by Q-PCR (Table 1). The results were evaluated as a Log_{10} reduction factor (LRF) in three different steps. The infectivity in filtrates of the antithrombin and haptoglobin samples was shown to be below the detection limit whereas the infectivity in the non-filtered control experiment done in parallel with the main filtration experiment was unchanged. No infectivity could be detected in samples of the IVIG solution before the filtration, most likely be derived from interference at low pH and/or by neutralization with anti-B19 IgG (Tsujikawa et al., 2011), because B19 DNA was detected in all the samples by Q-PCR (see Table 1).

3.2. Size distribution of B19 DNA in the filtrates of three different samples

The B19 DNA in the samples before and after filtration of the three plasma preparations was divided into five fractions by genome size using agarose gel electrophoresis. The B19 DNA in each fraction was quantified by Q-PCR and percentages of B19 genome amounts were determined (Fig. 1). The calculated percentage was considered to be only slightly influenced by the rate of recovery from each gel section because the rates were 54–85%. The majority of the fragments of B19 DNA in the three spiked process samples (start material) were the long chain (>4.0 kb fraction) including the full-length genome (approximately 5.6 kb) and less than 0.3% of the short chain (<0.5 kb fraction). In sharp contrast, the proportion of short chain B19 DNA were significantly increased to 84.8/93.3% in the sample after 15 nm filtration of the antithrombin sample, reflecting an enrichment of shorter chain fragments following filtration. The majority of the B19 DNA in the 19-nm filtrates of the haptoglobin and IVIG preparations was less than 1.0 kb. The percentage of the long chain B19 DNA in the antithrombin, haptoglobin, and IVIG sample after filtration was 2.0/0.5, 19.9/24.1, and 4.8/5.9%, respectively.

3.3. Reductions with different B19 DNA fragment sizes

The LRFs in different DNA size fractions in the three filtration steps were calculated from the amount of B19 DNA in each fraction (Table 2). For the 15-nm filtrate of the antithrombin sample and 19-nm filtrate of the haptoglobin sample, LRFs increased in proportion to DNA size and the short chain B19 DNA fragments were also removed to varying extents through the filters. In general, the LRFs in each fraction in the haptoglobin sample were lower than in the antithrombin sample. In the 19-nm filtrates of the IVIG sample, the LRF in the <0.5 kb and 0.5–1.0 kb fractions was approximately zero, and the LRFs in the more than 1.0 kb fractions increased with DNA size. Although the LRFs of the short chain B19 DNA were low, the LRFs of the long chain B19 DNA including full genomes in the

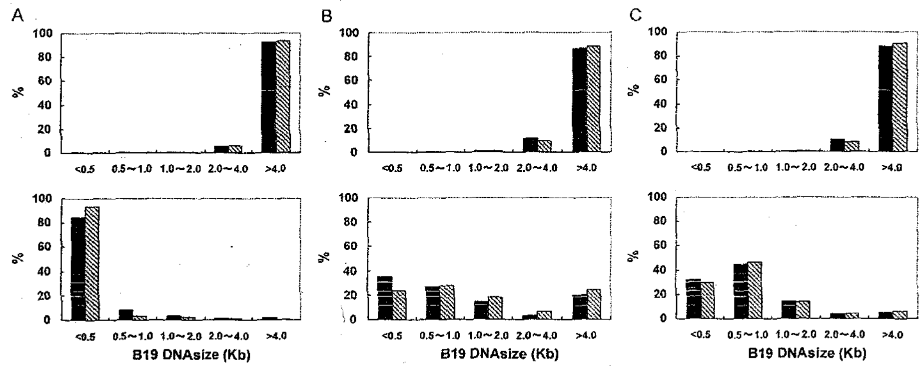

Fig. 1. Relative percentage of B19 genomes in different DNA size fractions in samples before (upper) and after (lower) filtration of three process samples of antithrombin, haptoglobin and IVIG products in duplicate. The 15-nm filtration of antithrombin (A), 19-nm filtration of haptoglobin (B), and 19-nm filtration of IVIG (C) are shown. The black and shaded bars show the percentages of run 1 and run 2, respectively.

Table 2
Reduction of B19 genomes in different DNA size fractions on three virus removal filtration steps.

	DNA size	<0.5	0.5–1.0	1.0–2.0	2.0–4.0	>4.0 kb
Antithrombin 15 nm	Pre	7.3/7.0	7.5/7.2	7.5/7.4	8.5/8.5	9.8/9.7
	Post	4.9/5.2	3.9/3.8	3.5/3.6	3.2/3.3	3.3/3.0
	Reduction	2.4/1.7	3.6/3.4	4.0/3.8	5.3/5.2	6.5/6.7
Haptoglobin 19 nm	Pre	5.8/6.3	6.5/6.6	6.7/6.9	7.8/7.7	8.7/8.7
	Post	4.8/4.8	4.7/4.9	4.5/4.7	3.8/4.2	4.6/4.8
	Reduction	1.0/1.4	1.8/1.7	2.3/2.2	4.0/3.4	4.1/3.9
IVIG 19 nm	Pre	5.7/5.2	6.1/5.8	6.7/6.2	7.8/7.3	8.7/8.3
	Post	5.8/5.7	6.0/5.9	5.5/5.3	4.9/4.8	5.0/5.0
	Reduction	−0.1/−0.4	0.1/0.0	1.2/0.8	2.9/2.5	3.7/3.4

B19 genome values are shown as Log_{10} IU/mL [Log_{10}(genome amounts in neat × (values in Fig. 1/100))].

antithrombin, haptoglobin and IVIG sample were 6.5/6.7, 4.1/3.9 and 3.7/3.4, respectively.

4. Discussion

The removal and/or inactivation of viruses during the manufacturing of biological products has been evaluated mainly using infectivity assays (ICH, 1997; EMEA, 1996; MHLW Japan, 1999). PCR may be of value in studies of processes which depend on the removal of viruses (EMEA, 1996), particularly where infectivity systems for a particular virus of concern are difficult to perform. Recently, Q-PCR assays were introduced to evaluate the removal of viruses by filtration, because they have a high precision and fast throughput (Lovatt, 2002). In addition, infectivity assays alone are unable to distinguish between removal and inactivation. Therefore, infectivity in combination with Q-PCR assays may provide more valuable information.

In one Q-PCR based study, viral genomes were detected in the sample after filtration, whereas the infectivity was below the detection limit using mouse minute virus and hepatitis A viruses (Kreil et al., 2006). In the present study, the infectivity in the filtrates of three different spiked products were below the detection limit, but the viral genome was detected in all the samples by Q-PCR. The result was positive when the amount of B19 genome determined with the Artus Kit was more than 5.3 Log_{10} IU/mL. The detection limit of the infectivity assay was approximately 4 Log_{10} lower than that of the Q-PCR assay. Therefore, whether the genomes detected by Q-PCR in filtrates are derived from full-genomes in infectious particles or degraded fragments of the genome was examined.

In this study, the size distributions of B19 DNA in filtrates using three process samples were examined. The LRF in each fraction of B19 DNA depended on the DNA fragment size. No reduction was shown in the samples with <1.0 kb of the IVIG filtrate. The degradation may be enhanced by hydrolysis at low pH. Interestingly, it was revealed that the proportion of degraded B19 DNA fragments in the filtrates of the three process samples was enhanced after 15 or 19-nm filtration. Notably, approximately 90% of B19 DNA in the 15-nm filtrate of the antithrombin sample was short chain DNA. Previously, it was reported that the LRF for 15-nm filtration using the antithrombin sample by PCR ELISA was 7.2 (Yunoki et al., 1999). PCR ELISA is a qualitative method for detecting B19 DNA by labeling amplicons with digoxigenin (DIG) (Yunoki et al., 2003). In contrast, the LRF for the same step was 4.8/4.5 ($n = 2$) by the Q-PCR method in this study. The short chain of B19 DNA in the 15-nm filtrate of the antithrombin sample detected by Q-PCR in this study (A 76 bp), but not by PCR ELISA (A 372 bp), may account for the difference in LRFs observed in the two studies. The size of PCR products obtained with the Artus kit in different fractions has been confirmed as 76 bp by agarose gel electrophoresis (data not shown). The detection limit for the short chain of B19 DNA by PCR ELISA was 2 $Logs_{10}$ lower than that of the long chain (data not shown). The significant difference in LRFs between Q-PCR and PCR ELISA could be explained by the enhanced ability to detect short chain DNA using the Q-PCR assay used in this study. The LRF for long chain B19 DNA in the filtration of the antithrombin sample was approximately 2 $Logs_{10}$ higher than that for un-fractionated samples by Q-PCR. On the other hand, the LRF determined by the PCR ELISA was close to the result of the long chain B19 DNA fraction by Q-PCR. The LRF determined by Q-PCR which can detect short chain DNA may therefore be underestimated, if the LRF from the long chain of B19 DNA reflects the capacity to remove infectious viral particles. A primer set which could amplify the long chain of DNA would therefore provide more relevant results in such instances. Primer sets for use to quantify longer DNA are currently under investigation in our laboratories. A primer set capable of amplifying approximately 1 kb of B19 DNA without a decrease in the detection limit has been obtained, although a primer set able to amplify >2 kb of B19 DNA without a decrease in the detection limit could not be established. Because large amounts of B19 DNA fragments of <1 kb exist in the filtrates of three process samples, a PCR system capable of quantifying approximately 1 kb of DNA might be effective for evaluation of viral removal. Therefore, attention should be paid to designing primers, especially to eliminate underestimation.

In conclusion, Q-PCR assays may be of use to evaluate viral removal, and complement infectivity assays. However, it should be noted that the result obtained by Q-PCR might underestimate the removal capacity of the manufacturing process where degraded fragments of viral genome may serve as targets in the Q-PCR assay. Hence the results of Q-PCR should be interpreted cautiously. Notably, the careful design of primers is needed to eliminate amplification by Q-PCR from viral DNA fragments.

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医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 2011年9月5日	新医薬品等の区分 該当なし。	総合機構処理欄
一般的名称	別紙のとおり。		研究報告の公表状況	MMWR.2011;60:1213-1215	公表国 米国	
販売名(企業名)	別紙のとおり。					

研究報告の概要

問題点：米国において、ブタインフルエンザA（H3N2）による熱性呼吸器疾患の患者から2009年に流行したインフルエンザA（H1N1）と考えられるウイルスとのリアソータント株が初めて同定された。

米国において、ブタインフルエンザA（H3N2）による熱性呼吸器疾患の患者2名から2009年に流行したインフルエンザA（H1N1）と考えられるウイルスとのリアソータント株が初めて同定された。2名とも入院後、回復した。1名はインディアナ州の男児で、ブタへの直接接触は確認されていないが、発症の2日前にブタと直接接触したケアワーカーと接触していた。男児の家族、男児との濃厚接触者、ケアワーカー、ケアワーカーの家族及びケアワーカーとの濃厚接触者に呼吸器系疾患に罹患した者は確認されなかった。もう1名はペンシルバニア州の女児で、農業祭におけるブタ及び他の動物への直接接触が確認された。女児の家族及び女児との濃厚接触者に罹患した者は確認されなかったが、農業祭参加者については調査中である。

2名の間に疫学的な関連は特定されておらず、現時点でヒトの追加症例は確認されていない。2名とも発症後の検査でブタインフルエンザA（H3N2）と同定されていたが、その後の全塩基配列決定で2009年に流行したインフルエンザA（H1N1）のものと考えられる配列が判明し、リアソータント株であることが示された。

使用上の注意記載状況・その他参考事項等

記載なし。

報告企業の意見	今後の対応
別紙のとおり。	今後とも関連情報の収集に努め、本剤の安全性の確保を図っていきたい。

10

MedDRA/J ver.14.0

別紙

一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免役グロブリン、⑤人免役グロブリン、⑥人免役グロブリン、⑦乾燥ペプシン処理人免疫グロブリン、⑧乾燥ペプシン処理人免疫グロブリン、⑨乾燥スルホ化人免疫グロブリン、⑩乾燥スルホ化人免疫グロブリン、⑪乾燥スルホ化人免疫グロブリン、⑫乾燥スルホ化人免疫グロブリン、⑬乾燥スルホ化人免疫グロブリン、⑭乾燥スルホ化人免疫グロブリン*、⑮乾燥濃縮人活性化プロテインC、⑯乾燥濃縮人血液凝固第Ⅷ因子、⑰乾燥濃縮人血液凝固第Ⅷ因子、⑱乾燥濃縮人血液凝固第Ⅷ因子、⑲乾燥濃縮人血液凝固第Ⅷ因子、⑳乾燥濃縮人血液凝固第Ⅸ因子、㉑乾燥濃縮人血液凝固第Ⅸ因子、㉒乾燥濃縮人血液凝固第Ⅸ因子、㉓乾燥濃縮人血液凝固第Ⅸ因子、㉔乾燥濃縮人血液凝固第Ⅸ因子、㉕乾燥濃縮人血液凝固第Ⅸ因子、㉖乾燥濃縮人血液凝固第Ⅸ因子、㉗乾燥抗破傷風人免疫グロブリン、㉘乾燥抗破傷風人免疫グロブリン、㉙抗HBs人免疫グロブリン、㉚抗HBs人免疫グロブリン、㉛トロンビン、㉜フィブリノゲン加第ⅩⅢ因子、㉝フィブリノゲン加第ⅩⅢ因子、㉞乾燥濃縮人アンチトロンビンⅢ、㉟乾燥濃縮人アンチトロンビンⅢ、㊱ヒスタミン加人免疫グロブリン製剤、㊲ヒスタミン加人免疫グロブリン製剤、㊳人血清アルブミン*、㊴人血清アルブミン*、㊵乾燥ペプシン処理人免役グロブリン*、㊶乾燥濃縮人アンチトロンビンⅢ
販売名(企業名)	①献血アルブミン20“化血研”、②献血アルブミン25“化血研”、③人血清アルブミン“化血研”*、④“化血研”ガンマーグロブリン、⑤ガンマーグロブリン筋注450mg/3mL「化血研」、⑥ガンマーグロブリン筋注1500mg/10mL「化血研」、⑦献血静注グロブリン“化血研”、⑧献血グロブリン注射用2500mg「化血研」、⑨献血ベニロンーⅠ、⑩献血ベニロンーⅠ静注用500mg、⑪献血ベニロンーⅠ静注用1000mg、⑫献血ベニロンーⅠ静注用2500mg、⑬献血ベニロンーⅠ静注用5000mg、⑭ベニロン*、⑮注射用アナクトC2,500単位、⑯コンファクトF、⑰コンファクトF注射用250、⑱コンファクトF注射用500、⑲コンファクトF注射用1000、⑳ノバクトM、㉑ノバクトM注射用250、㉒ノバクトM注射用500、㉓ノバクトM注射用1000、㉔ノバクトM静注用400単位、㉕ノバクトM静注用800単位、㉖ノバクトM静注用1600単位、㉗テタノセーラ、㉘テタノセーラ筋注用250単位、㉙ヘパトセーラ、㉚ヘパトセーラ筋注200単位/mL、㉛トロンビン“化血研”、㉜ボルヒール、㉝ボルヒール組織接着用、㉞アンスロビンP、㉟アンスロビンP500注射用、㊱ヒスタグロビン、㊲ヒスタグロビン皮下注用、㊳アルブミン20%化血研*、㊴アルブミン5%化血研*、㊵静注グロブリン*、㊶アンスロビンP1500注射用
報告企業の意見	インフルエンザウイルスは70～120nmの球形または多形性で、核酸は8本の分節状マイナス一本鎖RNA、エンベロープを有し、エンベロープの表面に存在する赤血球凝集素(HA)とノイラミダーゼ(NA)の抗原性により16種類のHA亜型および9種類のNA亜型に分類される。 今回の報告はブタインフルエンザA（H3N2）と2009年に流行したインフルエンザA（H1N1）とのリアソータント株を示唆する初めての報告であるが、ヒトに対し高病原性であるような情報は示されていない。 上記製剤の製造工程には、冷アルコール分画工程、ウイルス除去膜ろ過工程、加熱工程等の原理の異なるウイルスクリアランス工程が導入されており、各工程のウイルスクリアランス効果は「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（医薬発第1047号、平成11年8月30日）」に基づく、モデルウイルスを用いたウイルスプロセスバリデーションにより確認されている。今回報告したブタインフルエンザA（H3N2）ウイルスのモデルウイルスには、エンベロープの有無、核酸の種類等から、ウシウイルス性下痢ウイルス（BVDV）が該当すると考えられるが、上記工程のBVDVクリアランス効果については上記バリデーションにより確認されている。また、これまでに上記製剤によるブタインフルエンザA（H3N2）とインフルエンザA（H1N1）とのリアソータント株への感染報告例は無い。 以上の点から、上記製剤はインフルエンザに対する安全性を確保していると考える。

*：現在製造を行っていない

Swine-Origin Influenza A (H3N2) Virus Infection in Two Children — Indiana and Pennsylvania, July–August 2011*

On September 2, 2011, this report was posted as an MMWR *Early Release on the* MMWR *website (http://www.cdc.gov/mmwr).*

Influenza A viruses are endemic in many animal species, including humans, swine, and wild birds, and sporadic cases of transmission of influenza A viruses between humans and animals do occur, including human infections with avian-origin influenza A viruses (i.e., H5N1 and H7N7) and swine-origin influenza A viruses (i.e., H1N1, H1N2, and H3N2) (*1*). Genetic analysis can distinguish animal origin influenza viruses from the seasonal human influenza viruses that circulate widely and cause annual epidemics. This report describes two cases of febrile respiratory illness caused by swine-origin influenza A (H3N2) viruses identified on August 19 and August 26, 2011, and the current investigations. No epidemiologic link between the two cases has been identified, and although investigations are ongoing, no additional confirmed human infections with this virus have been detected. These viruses are similar to eight other swine-origin influenza A (H3N2) viruses identified from previous human infections over the past 2 years, but are unique in that one of the eight gene segments (matrix [M] gene) is from the 2009 influenza A (H1N1) virus. The acquisition of the M gene in these two swine-origin influenza A (H3N2) viruses indicates that they are "reassortants" because they contain genes of the swine-origin influenza A (H3N2) virus circulating in North American pigs since 1998 (*2*) and the 2009 influenza A (H1N1) virus that might have been transmitted to pigs from humans during the 2009 H1N1 pandemic. However, reassortments of the 2009 influenza A (H1N1) virus with other swine influenza A viruses have been reported previously in swine (*3*). Clinicians who suspect influenza virus infection in humans with recent exposure to swine should obtain a nasopharyngeal swab from the patient for timely diagnosis at a state public health laboratory and consider empiric neuraminidase inhibitor antiviral treatment to quickly limit potential human transmission (*4*).

Case Reports

Patient A. On August 17, 2011, CDC was notified by the Indiana State Department of Health Laboratories of a suspected case of swine-origin influenza A (H3N2) infection in a boy aged <5 years. The boy, who had received influenza vaccine in September 2010, experienced onset of fever, cough, shortness of breath, diarrhea, and sore throat on July 23, 2011. He was brought to a local emergency department (ED) where a respiratory specimen later tested positive for influenza A (H3). The boy was discharged home, but was not treated with influenza antiviral medications. He has multiple chronic health conditions, returned to the ED on July 24, 2011, and was hospitalized for treatment of those health problems, which had worsened. The boy was discharged home on July 27, 2011, and has since recovered from this illness. As part of routine CDC-supported influenza surveillance, the respiratory specimen collected on July 24, 2011, was forwarded to the Indiana State Department of Health Laboratories, where polymerase chain reaction (PCR) testing identified a suspect swine-origin influenza A (H3N2) virus on August 17, 2011. The specimen was forwarded to CDC where the findings were confirmed through genome sequencing on August 19, 2011.

No direct exposure to swine was identified for this child; however, a caretaker reported direct contact with asymptomatic swine in the weeks before the boy's illness onset and provided care to the child 2 days before illness onset. No respiratory illness was identified in any of the child's family or close contacts, the boy's caretaker, or in the family or contacts of the caretaker.

Patient B. On August 24, 2011, CDC was notified by the Pennsylvania Department of Health of a suspected case of swine-origin influenza A (H3N2) virus infection in a girl aged <5 years. The girl, who had received influenza vaccine in September 2010, experienced acute onset of fever, nonproductive cough, and lethargy on August 20, 2011. She was brought to a local hospital ED where a nasopharyngeal swab tested positive for influenza A by rapid influenza diagnostic test. She was not treated with influenza antiviral medications and was discharged home the same day. The girl has completely recovered from this illness.

A nasopharyngeal swab and nasal wash specimen were obtained at the ED and forwarded to the Pennsylvania State Department of Health Bureau of Laboratories for additional testing as part of routine CDC-supported influenza surveillance. On August 23, 2011, the state public health laboratory identified a suspected swine-origin influenza A (H3N2) virus by PCR testing, and both specimens were forwarded to CDC. On August 26, 2011, genome sequencing confirmed the virus as swine-origin influenza A (H3N2). On August 16, 2011, the girl was reported to have visited an agricultural fair where she had direct exposure to swine and other animals. No additional illness in the girl's family or close contacts has been identified, but illness in other fair attendees continues to be investigated. No additional confirmed swine-origin influenza virus infections have been identified thus far.

Epidemiologic and Laboratory Investigations

As of September 2, 2011, no epidemiologic link between patients A and B had been identified, and no additional cases of confirmed infection with the identified strain of swine-origin influenza A (H3N2) virus had been identified. Surveillance data from both states showed low levels of influenza activity at the time of both patients' illnesses. Case and contact investigations by the county and state human and animal health agencies in Indiana and Pennsylvania are ongoing, and enhanced surveillance for additional human cases is being implemented in both states.

Preliminary genetic characterization of these two influenza viruses has identified them as swine-origin influenza A (H3N2) viruses. Full genome sequences have been posted to publicly available web sites. The viruses are similar, but not identical to each other. Seven of the eight gene segments, including the hemagglutinin (HA) and neuraminidase (NA) genes, are similar to those of swine H3N2 influenza viruses circulating among U.S. pigs since 1998 (*2*) and previously identified in the eight other sporadic cases of human infection with swine-origin influenza A (H3N2) viruses in the United States since 2009.* The one notable difference from the viruses previously identified in human infections with swine-origin influenza A (H3N2) virus is that these two viruses have a matrix (M) gene acquired from the 2009 influenza A (H1N1) virus, replacing the classical swine M gene present in the prior eight swine-origin influenza A (H3N2) virus infections in humans.

Although reassortment between swine influenza and 2009 influenza A (H1N1) viruses has been reported in pigs in the United States (*3*), this particular genetic combination of swine influenza virus segments is unique and has not been reported previously in either swine or humans, based on a review of influenza genomic sequences publicly available in GenBank.† Analysis of data submitted to GenBank via the U.S. Department of Agriculture (USDA) Swine Influenza Virus Surveillance Program subsequent to this case identified two additional influenza A (H3N2) isolates from swine containing the M gene from the 2009 influenza A (H1N1) virus. Genome sequencing is underway to completely characterize the genetic composition of these two swine influenza isolates. (USDA Agricultural Research Service and USDA Animal and Plant Health Inspection Service, unpublished data, 2011).

The viruses in these two patients are resistant to amantadine and rimantadine, but are susceptible to the neuraminidase inhibitor drugs oseltamivir and zanamivir. Because these viruses carry a unique combination of genes, no information currently is available regarding the capacity of this virus to transmit efficiently in swine, humans, or between swine and humans.

Reported by

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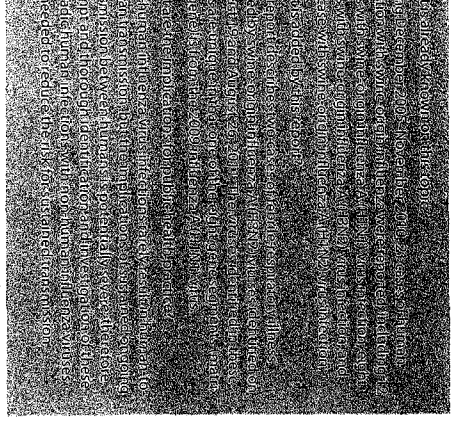
Editorial Note

To detect human infections with animal influenza viruses more effectively, CDC and state and local health departments have strengthened laboratory and epidemiologic procedures to promptly detect sporadic cases such as these. Since 2005, state public health laboratories have had the capability to detect non-human origin–influenza A viruses by PCR testing. From 2005 to 2007, CDC received reports of approximately one human infection with a swine-origin influenza virus each year. In 2007, human infection with a novel influenza A virus, including swine-origin influenza virus infections, became a nationally notifiable condition. Since that time, CDC has received approximately three to five reports a year of human infections with swine-origin influenza viruses. The recent increase in reporting might be, in part, a result of increased influenza testing capabilities in public health laboratories that allows for identification of human and swine-origin influenza viruses, but genetic changes in swine influenza viruses and other factors also might be contributing to this increase (*2,5,6*). During December 2005–November 2010, before the two cases described in this report, 21 cases of human infection with swine-origin influenza were reported (12 cases with swine-origin influenza A (H1N1) virus infection, eight cases with swine-origin influenza A (H3N2) virus infection, and one case

*Additional information is available at http://www.cdc.gov/flu/weekly/pastreports.htm.

†Available at http://www.ncbi.nlm.nih.gov/genbank.



with swine-origin influenza A (H1N2) virus infection). Six of these 21 cases occurred in patients who reported direct exposure to pigs; 12 patients reported being near pigs; human-to-human transmission was suspected in two cases after epidemiologic investigations revealed no reported contact with swine in either case, but contact with ill persons who reported swine exposure was the suspected source of infection; the exposure in one case was unknown (7) (CDC, unpublished data, 2011). Although the vast majority of human infections with animal influenza viruses do not result in human-to-human transmission (8,9), each case should be investigated fully to ascertain whether these viruses are transmitted among humans and to limit further exposure of humans to infected animals, if infected animals are identified. Such investigations require close collaboration between CDC, state and local public health officials, and animal health officials.

The lack of known direct exposure to pigs in one of the two cases described in this report suggests the possibility that limited human-to-human transmission of this influenza virus occurred. Likely transmission of swine-origin influenza A (H3N2) virus from close contact with an infected person has been observed in past investigations of human infections with swine-origin influenza A virus, but has not resulted in sustained human-to-human transmission. Preliminary evidence from the investigation of the Indiana case shows no ongoing transmission. No influenza illness has been identified, but if additional chains of transmission are identified, rapid intervention is warranted to try to prevent further spread of the virus. Clinicians should consider swine-origin influenza A virus infection as well as seasonal influenza virus infections in the differential diagnosis of patients with febrile respiratory illness who have been near pigs. Clinicians who suspect influenza virus infection in humans with recent exposure to swine should obtain a nasopharyngeal swab from the patient, place the swab in a viral transport medium, contact their state or local health department to facilitate transport and timely diagnosis at a state public health laboratory, and consider empiric neuraminidase inhibitor antiviral treatment (4). CDC requests that state public health laboratories send all suspected swine-origin influenza A specimens to the CDC, Influenza Division, Virus Surveillance and Diagnostics Branch Laboratory.

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別紙様式第2-1

No. 9

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
				2011. 9. 15	該当なし	
一般的名称	解凍人赤血球濃厚液		研究報告の公表状況	Sobata R, Matsumoto C, Igarashi M, Uchida S, Momose S, Hino S, Satake M, Tadokoro K. Transfusion. 2011 Sep;51(9):1949-56. doi: 10.1111/j.1537-2995.2011.03109.x. Epub 2011 Mar 17.	公表国 日本	
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社)					

研究報告の概要

○潜伏期間と推定される時期に献血した献血者に、新型インフルエンザ(パンデミック[H1N1]2009)血症は認められなかった

背景: 2009年春、新種のブタ由来インフルエンザA(H1N1)ウイルスが出現し、世界中で流行した。輸血由来と確認されたインフルエンザは報告されていないが、パンデミック(H1N1)2009の大流行は血液製剤の安全性に関して深刻な懸念を引き起こした。日本赤十字社血液センターではパンデミック(H1N1)2009感染疑いのある献血者から得られた血液製剤の供給を中止した。輸血によるパンデミック(H1N1)2009感染のリスクを調査するため、核酸増幅技術を用いて当該製品中のウイルス遺伝子検査を行った。

研究デザインと方法: 2009年6月から12月に、献血後7日以内にパンデミック(H1N1)2009と診断されたか強く疑われた579人の献血者から血液が採取され血液製剤が製造された。ウイルスRNAは血漿製剤と赤血球製剤から抽出し、パンデミック(H1N1)2009ウイルスの赤血球凝集素とマトリックス遺伝子のリアルタイムRT-PCRを実施した。

結果: 579人の献血者から計565の血漿製剤と413の赤血球製剤が得られた。579人の献血者のどのサンプルからもパンデミック(H1N1)2009のウイルスRNAは検出されなかった。

結論: 潜伏期間に献血したと思われる579人の献血者の中にパンデミック(H1N1)2009ウイルスが検出された者は1人もいなかった。輸血によるパンデミック(H1N1)2009の感染リスクは極めて低いと考えられる。

使用上の注意記載状況・その他参考事項等

解凍赤血球濃厚液「日赤」
照射解凍赤血球濃厚液「日赤」
解凍赤血球-LR「日赤」
照射解凍赤血球-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
パンデミック(H1N1)2009ウイルスの潜伏期間に献血をしたと思われる献血者の血液を調査したところ、ウイルスが検出された者は1人もおらず、輸血によるパンデミック(H1N1)2009ウイルス感染のリスクは極めて低いことが示唆されたとの報告である。	日本赤十字社では、問診で発熱などの体調不良者を献血不適としている。また、平成21年5月18日付薬食血発第0518001号「新型インフルエンザの国内発生に係る血液製剤の安全性確保について」に基づき、新型インフルエンザの患者又は罹患の疑いのある患者と7日以内に濃厚な接触があった人の献血を制限するほか、献血後に新型インフルエンザと診断された場合には当該製剤の回収と医療機関への情報提供を行うこととしていたが、平成21年度第4回血液事業部会運営委員会において、献血血液を通じて新型インフルエンザに感染する可能性が極めて低いことが示されたことから、平成22年3月5日に廃止された。今後も引き続き情報の収集に努める。

No viremia of pandemic (H1N1) 2009 was demonstrated in blood donors who had donated blood during the probable incubation period

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BACKGROUND: In the spring of 2009, the novel swine-origin influenza A (pandemic [H1N1] 2009) virus emerged and spread globally. Although no established cases of transfusion-transmitted influenza have been reported, the widespread outbreak of pandemic (H1N1) 2009 caused serious concern regarding the safety of blood products. The Japanese Red Cross Blood Centers have intercepted blood products with accompanying postdonation information indicating possible pandemic (H1N1) 2009 infection. To study the risk of transmission of pandemic (H1N1) 2009 by blood transfusion, we searched for the viral genome in such products using nucleic acid amplification technology.

STUDY DESIGN AND METHODS: Between June and December 2009, blood components were collected from 579 blood donors who were diagnosed as or strongly suspected of having pandemic (H1N1) 2009 within 7 days after donation. Viral RNA was extracted from plasma and red blood cell (RBC) products, and RNA samples were subjected to real-time reverse transcription–polymerase chain reaction of the hemagglutinin and matrix genes of the pandemic (H1N1) 2009 virus.

RESULTS: A total of 565 plasma and 413 RBC products from the 579 blood donors were available. No viral RNA of the pandemic (H1N1) 2009 was detected in any of the blood samples from the 579 blood donors.

CONCLUSION: No viremia of pandemic (H1N1) 2009 was demonstrated in any of the 579 blood donors who had most likely donated blood during the incubation period. It is considered that the risk of transmitting pandemic (H1N1) 2009 by blood transfusion is extremely low.

The novel swine-origin influenza A (pandemic [H1N1] 2009) virus was a triple-reassortant swine influenza virus that contains genes from human, swine, and avian influenza A viruses.[1-3] The pandemic (H1N1) 2009 virus emerged in early 2009 in Mexico and the United States[4,5] and rapidly spread worldwide including Japan[6] via human-to-human transmission because most people have no immunity to this new virus. Although no established cases of transfusion-transmitted influenza have been recognized and reported, the apparently high virulence reported in Mexico[7] raised a serious concern regarding the safety of blood products.

A few studies in the 1960s and 1970s have shown the viremia of seasonal influenza. Most data were obtained from blood samples that were collected after the onset of symptoms.[8-11] Only one instance of the detection of the virus in blood during the incubation period has been reported, but no virus has been detected from blood from the same individual at the onset of symptoms.[11] In recent reports, no viremia of seasonal influenza has been demonstrated.[12,13] Most studies have, thus, failed to demonstrate viremia in blood samples, but this is not unexpected considering that influenza is essentially a respiratory tract infection. There are almost no data on the viremia of pandemic (H1N1) 2009.

ABBREVIATIONS: HA = hemagglutinin; JRCBSs = Japanese Red Cross Blood Centers; M gene = matrix gene; NIID = Japanese National Institute of Infectious Diseases; PDI = postdonation information.

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TRANSFUSION 2011;51:1949-1956.

We previously reported that there was no viremia detected using nucleic acid amplification technology (NAT) in the blood samples obtained from 96 blood donors who showed symptoms of influenza within 7 days after the donation.[14] In this study, we report the result of our extended study in which 579 blood products from blood donors who were in the same presymptomatic period, as described above, were examined, together with the detailed profiles of such blood donors and the sensitivity of NAT used.

MATERIALS AND METHODS

Collection of blood

In May 2009, when the emergence of pandemic (H1N1) 2009 was confirmed in Japan, the Japanese Red Cross Blood Centers (JRCBCs) implemented the following measures to ensure the safety of blood products, in line with the notification from the Japanese Ministry of Health, Labour and Welfare: screening of blood donors for fever, refusing donation from blood donors who had returned to Japan from abroad within 4 weeks, and refusing donation from donors who had close contact with a patient suspected of having pandemic (H1N1) 2009 within 7 days.

From May 2009 to March 2010, the JRCBCs asked blood donors to provide postdonation information (PDI) on diagnosis of pandemic (H1N1) 2009 in addition to the usual information required. To acquire the PDI, the JRCBCs distributed handbills and explained to all blood donors in all blood collection sites: if they had any symptom of influenza within 7 days after blood donation, and it was diagnosed as pandemic (H1N1) 2009 in medical institutions, they were requested to contact the JRCBCs. If the PDI indicated a possible pandemic (H1N1) 2009 infection after the donation was given to the JRCBCs, the supply of blood products from such blood donors was stopped or withdrawn. These blood products collected from June to December 2009 were used for this study. After the blood products were collected from the inventory or retrieved from medical institutions, they were stored at −20°C in aliquots until use. The time lags between the donation and collection of the blood products for this study were 2 to 30 (mean, 10.3) days for plasma samples and 2 to 18 (mean, 7.8) days for red blood cell (RBC) samples. From the time of donation to collection, these blood products were stored at the stipulated temperatures (plasma, −20°C; RBCs, 4°C). Informed consent to tests for infection was obtained from all blood donors at the blood collection sites.

All blood donors in this study were diagnosed as having pandemic (H1N1) 2009 at a medical institution within 7 days after donation. These blood donors were classified into laboratory-confirmed cases and suspected cases on the basis of the confirmation standard for the diagnosis of pandemic (H1N1) 2009 infection. Laboratory-confirmed cases refer to those diagnosed as having pandemic (H1N1) 2009 by the reverse transcription–polymerase chain reaction (RT-PCR) using respiratory specimens in public health institutes. Suspected cases refer to those diagnosed as having pandemic (H1N1) 2009 infection by the rapid diagnostic kits for influenza A infection or on the basis of the symptoms of influenza-like illness such as fever and acute respiratory symptoms, without performing RT-PCR.

According to the Infectious Agents Surveillance Report published by the Japanese National Institute of Infectious Diseases (NIID), the pandemic (H1N1) 2009 virus dominated 99% of the influenza viruses isolated or detected from the cases of influenza-like illness during the study period from June to December 2009.[15] Therefore, suspected cases in this study were expected to be cases of either pandemic (H1N1) 2009 or noninfluenza illness, with negligible possibility of seasonal influenza.

Evaluation of NAT detection sensitivity

NAT detection sensitivity was evaluated by spiking experiments using virus particles of the pandemic (H1N1) 2009 virus (A/California/04/2009 [H1N1]) contained in the viral culture supernatant donated by NIID. The viral genome copy number of the culture supernatant was determined by quantitative RT-PCR, using synthesized RNA molecules of the matrix (M) gene as standards. The synthesized RNA was obtained from the cloned M gene inserted into plasmid DNA (TOPO TA cloning kit, Invitrogen, Carlsbad, CA) by transcription using T7 RNA polymerase (Roche Diagnostics, Indianapolis, IN). The transcribed RNA was purified using a commercially available kit (RNeasy Plus Mini Kit, Qiagen, Gaithersburg, MD), and its quantity and quality were checked using a capillary electrophoresis system (Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit, Agilent, Santa Clara, CA). A dilution series of the synthesized RNA sample was used to construct a standard curve to estimate the viral genome copy number of the culture supernatant.

The quantified culture supernatant of the pandemic (H1N1) 2009 virus was spiked into plasma and RBC samples from healthy volunteers, at inoculation doses from 20 to 2×10^5 genome equivalents (geq)/mL, and the NAT for the M and hemagglutinin (HA) genes was performed 20 times for each dose. The relationship between NAT positivity and pandemic (H1N1) 2009 virus concentration was analyzed by probit analysis. An input viral genome copy number with a 95% probability of a positive result was used as the detection limit.

NAT for detection of pandemic (H1N1) 2009 virus

Viral RNA was extracted from plasma and packed RBC samples using a kit for automated purification of viral

DNA and RNA (QIAamp Virus Biorobot MDx kit, Qiagen) and a viral nucleic acid purification kit (High Pure Viral Nucleic Acid Large Volume Kit, Roche Diagnostics), respectively. RNA samples were immediately subjected to the real-time RT-PCR of the M and HA genes of influenza A with a sequence detection system (PRISM 7900, Applied Biosystems, Foster City, CA) using an RT-PCR kit (Quanti-Tect Probe, Qiagen). The real-time RT-PCR of the HA gene was designed for the specific detection of the pandemic (H1N1) 2009 virus, whereas the real-time RT-PCR of the M gene was designed for the universal detection of type A influenza viruses. The sequences of the primers and probes used were synthesized in accordance with the protocols developed by NIID.[16] The forward and reverse primers were 5′-CCMAGGTCGAAACGTAYGTTCTCTCTA TC-3′ and 5′-TGACAGRATYGGTCTTGTCTTTAGCCAYTC CA-3′, respectively, for M gene and 5′-AGAAAAGA ATGTAACAGTAACACACTCTGT-3′ and 5′-TGTTTCCACAA TGTARGACCAT-3′, respectively, for HA gene. The TaqMan probes for M and HA genes were 5′-ATYTCGGCT TTGAGGGGGCCTG-3′ and 5′-CAATRTTRCATTTACC-3′, respectively. Each probe was labeled with a reporter dye (FAM) at the 5′ end, a nonfluorescent quencher and a minor groove binder at the 3′ end. Either 200 μL of plasma or 100 μL of RBC samples was used for each test. The real-time RT-PCR conditions comprised a 30-minute RT step at 60°C, a 10-minute initial PCR activation step at 95°C, and 45 amplification cycles at 95°C for 15 seconds and at 60°C for 45 seconds. To assess the analytical accuracy of NAT, a dilution series of pandemic (H1N1) 2009 virus particles in the viral culture supernatant as a positive control, and plasma and RBC samples obtained from healthy volunteers as negative controls were included in each assay.

Lookback investigation for blood recipients

As one of the operations in hemovigilance, JRCBCs have been collecting information on transfusion-transmitted infections and adverse transfusion reactions such as fever, urticaria, pain, nausea, hypotension, anaphylactic reaction or shock, dyspnea, and neuropsychiatric symptoms. If the blood products had already been released when the PDI indicating possible pandemic (H1N1) 2009 infection was acquired, we inform the medical institution of the blood product concerned. Patients who had received transfusion with the blood products involved with the pandemic (H1N1) 2009 infection were observed for influenza-like symptoms such as fever, respiratory symptoms, or systemic inflammatory reactions for a period of 7 days after transfusion.

RESULTS

Characteristics of blood donors

Between June and December 2009, the blood components were collected from 579 blood donors (314 male and 265 female). A total of 366 blood donors (190 male and 176 female) had laboratory-confirmed pandemic (H1N1) 2009 infection, and 213 blood donors (124 male and 89 female) had suspected pandemic (H1N1) 2009 infection.

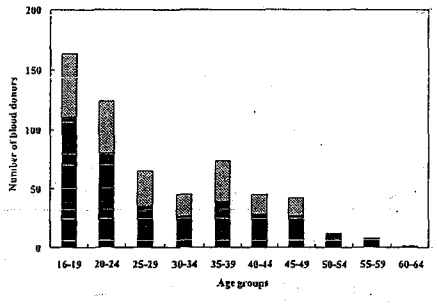

Fig. 1. Age distribution of the blood donors who had symptoms of pandemic (H1N1) 2009 after donation. Laboratory-confirmed cases (black bar) were diagnosed as pandemic (H1N1) 2009 by the RT-PCR method using respiratory specimens. Suspected cases (gray bar) were diagnosed by rapid diagnostic kits or on the basis of the symptoms of influenza-like illness, without performing RT-PCR. Sixty-one percent of the blood donors who showed symptoms of influenza after donation belonged to the young age group and were less than 30 years of age.

The ages of the 579 blood donors are shown in Fig. 1. Sixty-one percent of them were less than 30 years of age. The ratio of the donor number in the 16- to 29-year age group to the 579 blood donors were 2.2 times as high as the ratio of all blood donors in the 16 to 29 age group to the total number of donors who donated in 2009 in Japan. In each age group, there were no significant differences in the ratios between sexes (data not shown).

The time lag between the donation and the onset of influenza symptoms is shown in Fig. 2. Ten (1.7%) blood donors developed symptoms of influenza on the day of the donation, 74 (12.8%) within 1 day, 105 (18.1%) within 2 days, and 132 (22.8%) within 3 days after donation. That is, 321 (55.4%) blood donors showed symptoms of influenza within 3 days after donation.

The Infectious Agents Surveillance Report published by NIID indicated that the fall wave of pandemic (H1N1) 2009 appears to have peaked in late November 2009; this corresponded to the finding that 468 (80.8%) of the 579 blood donors donated between November and December 2009 (data not shown).

Evaluation of NAT detection sensitivity

For the plasma samples, the NAT showed a 100% detection probability for both M and HA genes at more than

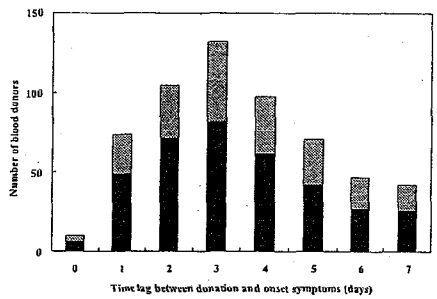

Fig. 2. Time lag between donation and onset of influenza symptoms. Laboratory-confirmed cases (black bar) were diagnosed as pandemic (H1N1) 2009 by the RT-PCR method. Suspected cases (gray bar) were diagnosed by rapid diagnostic kits or on the basis of the symptoms of influenza like illness, without performing RT-PCR. More than half of blood donors (55.4%) showed symptoms of influenza within 3 days after donation. It was speculated that many blood donors gave blood during the incubation period of the pandemic (H1N1) 2009 virus.

2000 geq/mL. The detection probabilities were 85 and 75% at 200 geq/mL and 50 and 15% at 20 geq/mL for the M and HA genes, respectively (Fig. 3A). For the RBC samples, the NAT showed a 100% detection probability for both M and HA genes at more than 20,000 geq/mL. The detection probabilities were 80 and 65% at 2000 geq/mL and 15 and 5% at 200 geq/mL for the M and HA genes, respectively (Fig. 3B).

The 95% detection limit of the NAT in the plasma samples was calculated to be 283 geq/mL (95% confidence interval [CI], 116-3287), corresponding to 57 geq per reaction (95% CI, 23-658) for the M gene, and 528 geq/mL (95% CI, 256-2368), corresponding to 106 geq per reaction (95% CI, 51-474) for the HA gene (Fig. 3A). For the RBC samples, the 95% detection limit of the NAT was calculated to be 3444 geq/mL (95% CI, 1784-12,697), corresponding to 344 geq per reaction (95% CI, 178-1270) for the M gene and 5292 geq/mL (95% CI, 2829-19,911), corresponding to 529 geq per reaction (95% CI, 283-1991) for the HA gene (Fig. 3B).

Detection of pandemic (H1N1) 2009 virus RNA

The NAT was performed using 565 plasma and 413 RBC samples obtained from 579 blood donors who showed symptoms of influenza within 7 days after donation. The samples consisted of 362 plasma and 271 RBC samples from the 366 blood donors who had laboratory-confirmed pandemic (H1N1) 2009 infection and 203 plasma and 142 RBC products from the 213 blood donors who had suspected pandemic (H1N1) 2009 infection. The NAT was performed in duplicate for the M and HA genes of the pandemic (H1N1) 2009 virus in each sample. None of the viral genome of the M or HA gene was detected in any of the plasma samples and RBC samples (Table 1).

Lookback investigation of blood recipients

In the lookback investigation of the donated blood products from the 579 blood donors, it was revealed that 36 platelet (PLT) products and 34 RBC products had already been used for transfusion when the PDI was acquired. Of the 36 blood donors who donated these PLT products, two showed symptoms of influenza on the next day of donation, and 10 and 13 showed symptoms 2 and 3 days after donation, respectively. Of the 34 blood donors who donated these RBC products, two and three showed symptoms of influenza 2 and 3 days after donation, respectively (Fig. 4). Of the blood donors who donated these PLT products and RBC products, 25 and 20 blood donors belonged to laboratory-confirmed cases, respectively.

In the 70 blood recipients who received blood products likely donated during the incubation period of the pandemic (H1N1) 2009 infection, influenza-like symptoms such as fever and acute respiratory symptoms and any transfusion adverse reactions were not observed for a period of 7 days after transfusion.

DISCUSSION

In this study, we examined blood samples from 579 blood donors who were diagnosed as or strongly suspected of having pandemic (H1N1) 2009 infection within 7 days after donation. Sixty-one percent of the blood donors involved in this study belonged to the young age group of less than 30 years of age. The ratio of the young age group to the 579 blood donors was higher than that of this age group to the total number of blood donors who donated in 2009 in Japan. It has been reported that the majority of patients with pandemic (H1N1) 2009 were children and young people.[17,18] According to the reports by the Centers for Disease Control and Prevention in the United States, more than 64% of the pandemic (H1N1) 2009 virus-infected individuals were 5 to 24 years old; only 1% were 65 years of age or older.[17] In this point, pandemic (H1N1) 2009 markedly differs from seasonal influenza. The ratio of the age groups of the blood donor cohort involved in this study reflected the ratio of the age groups of the pandemic (H1N1) 2009 virus-infected individuals reported in Japan and abroad.[17,19]

In this study, 10 blood donors showed symptoms of influenza on the day of the donation, 74 within 1 day, and 105 within 2 days after the donation. The incubation

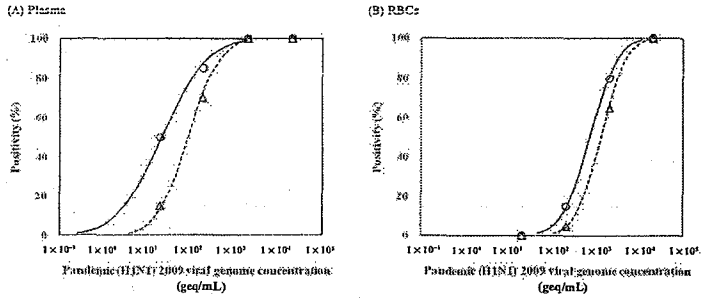

Fig. 3. Sensitivities of the NAT for plasma samples (A) and RBC samples (B). The quantified culture supernatant of the pandemic (H1N1) 2009 virus was spiked into the plasma and RBC samples from healthy volunteers, at doses from 20 to 2×10^5 geq per mL, and the NAT for the M (—) and HA (- - -) genes was performed 20 times for each concentration. The relationship between NAT positivity and pandemic (H1N1) 2009 virus concentration was analyzed by probit analysis. The 95% detection limit of the NAT for the plasma samples was calculated to be 283 geq/mL for the M gene and 528 geq/mL for the HA gene (A). For the RBC samples, the 95% detection limit of the NAT was calculated to be 3444 geq/mL for the M gene and 5292 geq /mL for the HA gene (B).

TABLE 1. Results of detection of pandemic (H1N1) 2009 virus RNA in plasma or RBCs drawn from blood donors who were diagnosed as pandemic (H1N1) 2009 after donation

Cases	Total number of blood donors	Number of samples tested		NAT-positive samples			
				Plasma		RBCs	
		Plasma	RBC	M gene	HA gene	M gene	HA gene
Laboratory-confirmed case*	366	362	271	0	0	0	0
Suspected case†	213	203	142	0	0	0	0
Total	579	565	413	0	0	0	0

* Pandemic (H1N1) 2009 was diagnosed by the RT-PCR method using respiratory specimens.
† Pandemic (H1N1) 2009 was diagnosed by the rapid diagnostic kits for influenza A or on the basis of the symptoms of influenza-like illness, without performing RT-PCR.

period of the pandemic (H1N1) 2009 virus is reported to be 1 to 7 days, with a mean of 2 days.[5,20] Therefore, it is speculated that many of the 579 blood donors gave blood during the incubation period of the pandemic (H1N1) 2009 virus. NAT was performed using specimens of this donated blood. No RNA of the pandemic (H1N1) 2009 virus was detected in any of the blood samples; no viremia of pandemic (H1N1) 2009 before clinical onset was demonstrated. In this study, however, some donors included in the 213 suspected cases were diagnosed by physicians on the basis of the symptoms of influenza-like illness without performing RT-PCR or rapid diagnostic tests. The possibility that donors with noninfluenza illness were not completely excluded from the suspected cases would weaken the power of this study.

Although influenza epidemics occur every winter season, no established cases of transfusion-transmitted influenza have been recognized and reported. A few studies published from the 1960s to the 1970s showed the presence of viremia of seasonal influenza;[8-11] however, among studies published in recent years, no viremia of seasonal influenza has been demonstrated yet.[12,13] In both pandemic (H1N1) 2009 and seasonal influenza virus infections, the peak viral load in the respiratory specimens was observed immediately after the onset of symptoms.[21,22] Although the mean viral load in the respiratory specimens of pandemic (H1N1) 2009 was 1.84×10^8 copies/mL, the virus was detected in none of the blood specimens obtained at the same time.[20] In addition to these data, we detected no viremia of pandemic (H1N1) 2009 in the present study. It thus seems that the occurrence of pandemic (H1N1) 2009 viremia before the onset of illness is extremely low. If there were cases in which influenza infections occurred by blood transfusion, a viremia condition would need to be present during the incubation period in which blood donation was performed. It is thus considered that the risk of the transmission of the pandemic (H1N1) 2009 virus by blood transfusion is extremely low.

The 95% detection limit of NAT for the plasma samples was calculated to be 283 geq/mL for the M gene and 528 geq/mL for the HA gene, whereas that for the RBC samples was calculated to be 3444 geq/mL for the M gene and 5292 geq/mL for the HA gene. The NAT sensitivity for the HA gene was lower than that for the M gene, for both plasma and RBC samples. This difference could be due to the secondary structure of the viral RNA or reverse-transcribed cDNA that might have decreased the sensitivity of the RT-PCR of the HA gene. The lower sensitivity in the packed RBC samples might be caused by the inhibitors of RT-PCR such as hemoglobin that contaminated the RNA solutions obtained from the RBC samples or the insufficient efficiency of the viral RNA extraction. Improved methods will be required to purify viral RNA from RBC samples. If low-level viremia below the NAT detection sensitivity should exist, it would not be detected using our assay.

In the first step in influenza infection and viral replication, influenza viruses bind through the HA transmembrane glycoprotein onto sialic acid residues on the surface of epithelial cells, typical in respiratory organs. After HA is cleaved by a protease, the cells import the virus by endocytosis.[23-25] HA cleavage is required to activate virus infectivity, and the activating proteases are mainly distributed in the respiratory organs and intestine in humans,[24,26] so that it should be difficult for influenza viruses to acquire infectivity in blood. In theory therefore the risk of the direct transmission of influenza via blood is considered to be extremely low. In fact, we showed, in this study, that the transfusion of 36 PLT and 34 RBC products from the blood donors who likely donated during the incubation period of the pandemic (H1N1) 2009 virus caused apparently no transmission of the virus.

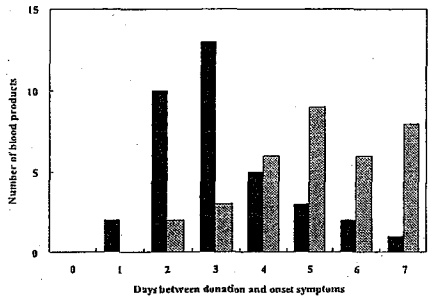

Fig. 4. Time lag between donation of blood implicated in lookback study and onset of symptoms. Thirty-six PLT products (black bar) and 34 RBC products (gray bar) likely donated during the incubation period of the pandemic (H1N1) 2009 had already been used for transfusion to 70 recipients when the PDI was acquired. In the lookback investigation, no influenza-like symptoms or any other observable symptoms were observed in the recipients after transfusion.

The risk of virus infection by blood transfusion has decreased owing to the introduction of a screening test for donated blood, but there is still residual risk caused by pathogens that are excluded from targets of the current screening test or newly spread in humans. The influenza virus is one of the potentially unrecognized pathogens in the blood supply. This study showed that the viremia of pandemic (H1N1) 2009 during the incubation period is highly unlikely to occur and that it does not pose a noticeable risk to the safety of the blood supply. The main infection routes of the influenza virus are droplet infection and contagious infection. During a pandemic, many people are easily infected by the influenza virus. Compared with the risk of infection by the influenza virus via respiratory droplets, the risk of transmission by transfusion is almost negligible. In this point, pandemic (H1N1) 2009 markedly differs from any other currently known viral threats to the blood supply.

Regarding influenza viruses with high pathogenicity, we do not know the risk of their transmission by transfusion. The HA protein of highly pathogenic avian influenza virus can be cleaved by proteases that are produced in many different tissues. As a results, these viruses can replicate in many organs of the bird, not just the respiratory organs.[27] In severe cases of highly pathogenic avian influenza A (H5N1) virus-infected humans, viremia has been reported.[28-30] New studies will be required when a new type of influenza emerges in the future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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別紙様式第２－１

医薬品　研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
一般的名称	－		研究報告の公表状況	Published Online September 22 2011 Science 11 November 2011: Vol. 334 no. 6057 pp. 814-817 DOI: 10.1126/science.1213841	公表国 米国	
販売名(企業名)	－					

研究報告の概要	使用上の注意記載状況・その他参考事項等
ゼノトロピック MLV 関連ウイルス(xenotropic-MLV-related virus：XMRV)を含むマウス白血病ウイルス(MLV)が慢性疲労症候群(CFS)に関連していることが議論されている。 この問題を更に深く検討するため、以前に XMRV/MLV 陽性と報告された被験者 15 例(14 例が CFS 患者)および以前に XMRV/MLV 陰性と判定された健康ドナー15 例から採取した血液サンプルを収集した。 これらのサンプルを二重盲検下で 9 ヵ所の研究所に分配し、XMRV/MLV 核酸、ウイルス複製および抗体を検出する検査を行った。 現在利用可能な XMRV/P-MLV 検査法は、以前に陽性結果を報告した 3 ヵ所の参加研究所が採用している検査法を含め、CFS 患者および対照から採取したサンプルにおいて直接的ウイルスマーカー(RNA、DNA または培養物)あるいは特異的抗体を再現性よく検出できないと結論付けられた。 今回の知見により、現時点では血液提供者への通常のスクリーニングに XMRV/P-MLV に関する検査を実施する正当な理由はないことが示唆された。	

報告企業の意見	今後の対応
慢性疲労症候群との関連が疑われる XMRV やその他の MLV に対する血液スクリーニングの要否に関する情報であるが、検査の再現性が担保できず、現時点ではスクリーニング項目として採用する正当な理由はないとの情報であった。	今後とも XMRV やその他の MLV に関する安全性情報等に留意していく。

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Report

Failure to Confirm XMRV/MLVs in the Blood of Patients with Chronic Fatigue Syndrome: A Multi-Laboratory Study

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†A description of the SRWG is available as supporting material in *Science* Online.

Murine leukemia viruses (MLV), including xenotropic-MLV-related virus (XMRV), have been controversially linked to chronic fatigue syndrome (CFS). To explore this issue in greater depth, we compiled coded replicate samples of blood from 15 subjects previously reported to be XMRV/MLV-positive (14 with CFS) and from 15 healthy donors previously determined to be negative for the viruses. These samples were distributed in a blinded fashion to nine laboratories which performed assays designed to detect XMRV/MLV nucleic acid, virus replication, and antibody. Only two laboratories reported evidence of XMRV/MLVs; however, replicate sample results showed disagreement and reactivity was similar among CFS subjects and negative controls. These results indicate that current assays do not reproducibly detect XMRV/MLV in blood samples and that blood donor screening is not warranted.

Novel murine leukemia virus (MLV)-like sequences were identified in, and implicated as a potential infectious cause of, human prostate cancer in 2006 (*1*). These sequences appeared to be closely related to xenotropic MLV (X-MLV) and were termed X-MLV-related virus or XMRV. In 2009, similar viral sequences were identified in a cohort of patients with chronic fatigue syndrome (CFS) (*2*). In that study XMRV could be directly cultured from both peripheral blood mononuclear cells (PBMC) and plasma from the majority of patients with CFS, and XMRV sequences were detected by PCR and RT-PCR (*2*, *3*). Furthermore, evidence of an immune response to MLVs was observed in patient plasma (*2*, *3*). In an *independent study, other patients with CFS were reported to* harbor MLV-related virus sequences, but not XMRV, in PBMC and plasma (*4*). These sequences were derived from viruses resembling polytropic MLVs (P-MLV), rather than X-MLV. Importantly, both studies identified XMRV/P-MLV in the majority (67 to 86%) of patients with CFS but also in substantial numbers of healthy controls including blood donors (4-7%) (*2*, *4*).

Subsequent studies cast doubt on the association between XMRV/P-MLVs and CFS, and indeed on the detection of XMRV/P-MLVs in human populations (reviewed in (*5*)). Many, although not all (*6*, *7*), of these negative studies focused on nucleic acid detection and/or serology and did not include cell culture assays for virus (*8–11*). Several additional findings raised uncertainty about the high rates of XMRV/P-MLV in patients with CFS that had been described in the two seminal papers: (i) clinical samples and PCR reagents were found to be contaminated by XMRV and mouse DNA containing endogenous MLVs (*12*); (ii) XMRV and P-MLV lack the sequence diversity that would be expected to arise following transmission, infection, and repeated cycles of replication of a retrovirus in humans (*13*, *14*), and (iii) evidence was presented which strongly suggested that XMRV originated in the early 1990s by recombination of endogenous

MLVs following serial passage of a human prostate xenograft in laboratory mice (*15*). It was postulated that this laboratory passage resulted in the generation of several prostate cancer cell lines harboring integrated XMRV sequences that produced high levels of infectious virions. These XMRV-infected cell lines were subsequently widely disseminated and likely produced inadvertent XMRV contamination of laboratories and reagents (*15*).

We report here the results of a comprehensive study where multiple laboratories analyzed the same blood samples for XMRV/P-MLV. These blood samples, which were drawn from persons who were previously reported to be XMRV- (*2*) or P-MLV-positive (*4*) and from blood donors who previously tested negative for XMRV, were aliquoted into replicate tubes and assembled into coded panels together with replicates of experimentally prepared positive control samples. The testing was performed fully blinded to remove bias. These samples were tested by nine laboratories using highly sensitive and previously validated nucleic acid, serological and culture assays (tables S1 to S5) for XMRV and other MLVs (*16*). The two laboratories that had previously found an association for the MLVs with CFS participated in this study (*2, 4*). All nine laboratories used XMRV/P-MLV nucleic acid amplification testing (NAT), serological and/or culture assays of their own choosing which were incorporated into parallel or serial testing algorithms to generate final results. The majority of laboratories included assays to detect murine DNA contamination either on all samples or on all NAT positive samples.

Fourteen patients with CFS, together with one person reporting contact with a CFS patient [described in supporting online material (*17*)], all of whom were previously reported to be XMRV/P-MLV-positive by at least one method (table S6) were enrolled into the study at two clinical sites using IRB-approved protocols and consents (referred to as the XMRV/P-MLV cohorts henceforth). Per study protocol, none of the 15 subjects were on antiretrovirals, but several later disclosed that they were taking other antivirals (e.g., valacyclovir) and two were on immunosuppressive medications (the latter are indicated in table S6). In the case of the P-MLV-like viruses described by Lo and colleagues (*4*), only PCR detection had been performed in the original study; four of five patients enrolled into the current study were reported to be P-MLV reactive on the archived samples from the original cohort study and on a second sample collected 15 years later (2010) whereas one patient was PCR-positive only on the original archived sample (*4*). The Whittemore Peterson Institute (WPI) patient cohort was more intensively characterized as positive by PCR, serology and/or culture, although none of the study subjects tested positive in all assays at all time points (table S6).

To minimize introduction of potential contaminants, we took extensive precautionary measures during the collection of specimens and the laboratory processing of blood and preparation of sample aliquots (*17*). Blood specimens were collected by independent phlebotomists, shipped to the central laboratory (*17*), and processed into coded PBMC, plasma and whole blood (WB) aliquots. Similarly, fifteen control specimens from blood donors (n=12) or laboratory controls (n=3) that had been established as negative for XMRV and MLVs by PCR, serology and culture by multiple laboratories, were collected, processed and aliquoted in parallel (*17*). Finally, a separate facility in the central laboratory prepared and characterized stocks of the XMRV-infected human cell line 22Rv1 (*15, 18*) and supernatant, which were used to spike samples to create a set of low-level positive controls (*17*).

A total of eleven NAT, five serology and three culture assays were performed on the samples (*17*). The WPI laboratory did not report culture assay results because their target cells had become contaminated with mycoplasma. Other than this, all sites reported results on all distributed and coded sample aliquots to the central laboratory. The results were then decoded and compiled into analysis datasets specific to the panels.

Few positive NAT results were reported, other than on the coded spiked positive control replicate aliquots (table 1) (table S7). Six of seven laboratories that performed NAT on three sample types (plasma, PBMC and WB) reported no positive result for coded clinical samples (XMRV/P-MLV cohorts or negative controls), whereas these laboratories detected XMRV in 100% of the spiked controls (table 1). These laboratories included those that employed the most sensitive XMRV/P-MLV assays available, based on our previous blinded analytical sensitivity performance study (*16*). Of particular note, the FDA/Lo laboratory failed to detect MLV-like sequences using the same nested PCR assay as previously published, in either the known negative controls or in the XMRV/P-MLV cohort samples. The samples scored as negative by this laboratory included the replicate samples from five patients with CFS reported as P-MLV positive in their previous study, four of whom had also tested positive on a second specimen collected over a decade after the archived CFS cohort panel (*4*).

The only positive NAT results on some of the replicates from clinical samples were reported by WPI. The WPI assays appeared less sensitive than those used by the other laboratories, based on the fact that only 3 of 5 plasma and 4 of 5 PBMC-spiked positive control replicates were scored as positive by WPI (table 1) (table S7). However, two plasma clinical aliquots were reported as positive in the WPI nested RT-PCR *gag* assay. These samples were from two different negative controls, and only one out of the three replicates was positive in each case. Sequencing of the excised bands revealed 1-3 base changes compared to XMRV derived from 22Rv1 (supporting online text). A clinical PBMC sample, derived from one of the nine WPI CFS patients, was also positive in WPI's nested *gag* PCR assay. However, only one of two PBMC replicates for this individual was positive, and all replicates of plasma and WB from this patient were reported as negative by WPI. All positive samples tested negative for mouse DNA contamination as assessed by mouse mitochondrial DNA PCR (*4*). Reactivity rates did not significantly differ between samples from negative controls and the XMRV/P-MLV cohorts ($p > 0.05$) (supporting online text, table S10).

In the initial study, Lombardi et al. reported that the most effective and consistent method of determining whether an individual was XMRV-positive was by isolation of replication-competent virus through co-culture of target prostate cell lines with either patient PBMCs or plasma (*2, 3*). Although culture results were not reported by WPI in the present study, the NCI/Ruscetti laboratory also successfully performed virus culture using both plasma and PBMC in the Lombardi et al. study (*2, 3*). Additionally, virus culture was performed by the FDA/Hewlett laboratory, which used two methods, one of which (LNCaP cell culture) was established in their laboratory for this study based on WPI procedures and on-site training by the lead investigators from the WPI and NCI/Ruscetti laboratories, and hence viral culture in this laboratory would be expected to have equivalent sensitivity to the culture method used by Lombardi et al. (*17*). Both laboratories successfully detected all five replicates of the spiked positive controls (~10^6 RNA copies/ml). However, while neither of the FDA/Hewlett assays detected confirmed positive cultures in the 30 coded clinical aliquots, the NCI/Ruscetti laboratory reported nine aliquots as positive (table 1, 2). Six of the positive results were from negative control samples (40% positive rate); these six subjects/samples had previously been pedigreed by the same laboratory as culture-negative (*17*). In contrast, only three (20%) of the 15 XMRV/P-MLV-cohort subjects (including ten subjects who had previously been found to be culture-positive by the WPI and NCI/Ruscetti laboratories) tested positive in the coded panel (table S1). There was no significant difference between the rate of reported positive culture results among negative controls and the XMRV/P-MLV cohort subjects (p-value = 0.43, table S8).

Finally, serology was performed by four laboratories (*17*). Although plasma with human antibodies to XMRV/P-MLVs was not available to produce spiked controls for serology, all four laboratories performed their own internal controls (*17*). Three assays --a Western blot test using purified XMRV (CDC) (*19*) and two chemiluminescent immunoassays using recombinant XMRV gp70 and p15E (Abbott Diagnostics) (*20*)-- failed to detect positive results for any of the coded replicates prepared from the 30 clinical samples. A flow cytometry-based serologic assay run by two laboratories (NCI/Ruscetti and WPI), utilizing mouse cells expressing the spleen focus-forming virus (SFFV) envelope as employed in the original Lombardi et al. study, reported a number of positive results on samples from both the XMRV/P-MLV cohorts and the negative-plasma controls. The NCI/Ruscetti laboratory reported 13 positive samples, including eight (53%) from 15 known negatives and five (33%) from 15 XMRV/P-MLV cohort subjects (table 1) (table 2). None of the positive results from the XMRV/P-MLV cohorts or controls were reported for more than one of the uniquely coded replicates, despite the fact that every sample was represented in the panel in duplicate or triplicate (table 2). There was no significant difference between the proportions of negative controls and XMRV/P-MLV cohort subjects identified as serology-positive (p-values >0.20 regardless of how positivity was defined [supporting online text, table S9]).

Among all serologic replicates tested, the WPI detected 22 positives, including 10 reactive results among the negative controls, and six each in the subjects previously reported as positive by WPI and by FDA/Lo (table 1) (table S7). Three of the six known negative controls with a positive serology result had at least two of three replicates positive (table 2). All five patients previously identified as P-MLV positive by FDA/Lo had a replicate called serology positive, but only one had both replicates reported as positive. Similarly for the 10 subjects previously identified as XMRV positive by WPI, four subjects had one of two replicates reported as serology positive, while both replicates from one patient were reported positive (table 2). There was no significant difference in the rates of positive WPI serology results between negative controls and XMRV/P-MLV cohort subjects (p-value = 0.27). There was no statistical agreement between the samples reported as serology positive by the NCI/Ruscetti and WPI laboratories, despite the fact that they used similar assays (supporting online text, tables S9, S10). Kappa values were calculated for each criterion and for all subjects combined using standard procedures (*17, 21*). The Kappa values for level of agreement of results between these two laboratories ranged from -0.20 for WPI XMRV/P-MLV-positive subjects (no agreement) to 0.21 for all negative controls combined (fair agreement). However, the most telling Kappa value between the WPI and NCI/Ruscetti serology results is the one computed for all subjects combined, which is 0.01 indicating no agreement.

In summary, our study demonstrates that no XMRV/P-MLV assay in any of the nine participating laboratories could reproducibly detect XMRV/P-MLV in fifteen subjects (fourteen with CFS) who had previously been reported as XMRV/P-MLV-infected usually at multiple time points and

often by multiple assays (*2*, *4*). The two laboratories (WPI and NCI/Ruscetti labs) that reported positive results in this study reported similar rates of reactivity among XMRV/P-MLV subjects and known negative control donor samples. The results from both laboratories were inconsistent when their assays were performed in parallel on replicate sample aliquots derived from individual subject specimens. There was also no agreement of reactivity when comparing results between these two laboratories for the 30 blinded XMRV/P-MLV cohorts and control samples. In contrast, assays developed by FDA (Lo and Hewlett), CDC, NCI/DRP, Abbott Diagnostics, Abbott Molecular and Gen-Probe, all of which have been designed to detect XMRV and relevant MLVs with high sensitivity and specificity, failed to detect evidence of viral infection in any of the previously positive subjects, including CFS patients, or negative control specimens represented in the study.

Altogether, 15 XMRV/P-MLV cohort subjects were represented in this study, the maximum number of subjects who could be recruited by the cohort investigators (*2*, *4*). Since most patients were selected based on having previously tested positive for XMRV/P-MLV 1-3 years ago, it is possible that levels of viremia and/or antibody could have waned by the time samples were drawn in our study; however, this is contradictory to Lo et al.'s finding that 4 of 5 patients retested positive 15 years later (*4*). The inconsistent reactive results from the two laboratories that previously reported detection of XMRV (NCI/Ruscetti and WPI) and the negative results from all other laboratories, including the laboratory that previously reported detection of P-MLV (FDA/Lo), strongly suggest that the positive reactivity in this study represents false positive results due to assay non-specificity or cross-reactivity (e.g. to other endogenous or exogenous retroviruses). However, we cannot definitively exclude the possibility that the levels of XMRV/P-MLV markers in blood may be at or below the limit of detection of all assays and/or fluctuate over time as recently described in experimentally infected macaque studies (*22*).

Based on these findings, we conclude that currently available XMRV/P-MLV assays, including the assays employed by the three participating laboratories that previously reported positive results on samples from CFS patients and controls (*2*, *4*), cannot reproducibly detect direct virus markers (RNA, DNA, or culture) or specific antibodies in blood samples from subjects previously characterized as XMRV/P-MLV positive (all but one with a diagnosis of CFS) or healthy blood donors. Finally, our findings are reassuring with respect to blood safety and indicate that routine blood donor screening for XMRV/P-MLV is not warranted at this time.

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screening assays for pathogen nucleic acids. S. K. is a paid consultant to Novartis Diagnostics, a distributor of blood donor screening assays, and to Cerus Corporation, a manufacturer of pathogen inactivation systems for blood components. The Whittemore Peterson Institute has filed patent applications related to methods of testing XMRVs and variants in blood. Abbott Laboratories has filed patent applications relating to detection of XMRV using immunoassays and molecular-based assays. Gen-Probe has filed patent applications relating to the assays they performed in this paper.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1213841/DC1
Materials and Methods
SOM Text
Tables S1 to S10
References (*23–35*)
Appendix S1

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Table 1. All XMRV/P-MLV assay results from all laboratories.

Test	Laboratory	Sample type: Negative Controls*	WPI XMRV/P-MLV Subjects*	Lo et al. XMRV/P-MLV Subjects*	Spiked Controls*
NAT/Plasma	Abbott-M†	0/15	0/10	0/5	5/5
	CDC	0/15	0/10	0/5	5/5
	FDA/Lo	0/15	0/10	0/5	5/5
	FDA/Hewlett	0/15	0/10	0/5	5/5
	Gen-Probe	0/15	0/10	0/5	5/5
	NCI/DRP	0/15	0/10	0/5	5/5
	WPI	**2/15‡**	0/10	0/5	3/5
NAT/PBMC	Abbott-M	0/3	0/10	0/5	5/5
	CDC	0/3	0/10	0/5	5/5
	FDA/Lo	0/3	0/10	0/5	5/5
	FDA/Hewlett	0/3	0/10	0/5	5/5
	Gen-Probe	0/3	0/10	0/5	5/5
	NCI/DRP	0/3	0/10	0/5	5/5
	WPI	0/3	1/10‡	0/5	3/5
NAT/WB	Abbott-M	0/15	0/10	0/5	5/5
	CDC	0/15	0/10	0/5	5/5
	FDA/Lo	0/15	0/10	0/5	5/5
	FDA/Hewlett	0/15	0/10	0/5	5/5
	Gen-Probe	0/15	0/10	0/5	5/5
	NCI/DRP	0/15	0/10	0/5	5/5
	WPI	0/15	0/10	0/5	5/5
Culture	FDA/Hewlett	0/15	0/10	0/5	5/5
	NCI/Ruscetti	**6/15**	**3/10‡**	0/5	5/5
Serology	Abbott-D	0/15	0/10	0/5	N/A
	CDC	0/15	0/10	0/5	N/A
	NCI/Ruscetti	8/15	3/10	2/5‡	N/A
	WPI	**6/15**	**5/10**	5/5‡	N/A

*Number positive/number tested. A single reactive replicate out of 1, 2, or 3 tested for a given individual was considered positive
†Abbott-M, Abbott Molecular; Abbott-D, Abbott Diagnostics; WB, whole blood; N/A, not applicable
‡No significant association was seen when the reactivity rates of control negatives and XMRV/P-MLV cohort subjects were compared [*P* values are discussed (supporting online material text)]

Table 2. Results of replicates for assays with positive results (number reactive/number replicates tested).

Sample Type	Subject	Assay	WPI NAT/Plasma	WPI NAT/PBMC	WPI Serology	NCI/Ruscetti Serology	NCI/Ruscetti Culture
Negative Controls	1		0/1	0/2	0/2	0/2	0/1
	2		0/1	0/1	0/2	0/2	1/1
	3		0/1	0/1*	1/2	1/2	0/1
	4		0/3	NT*	1/3	1/3	0/1
	5		0/3	NT	0/3	0/3	0/1
	6		1/3	NT	0/3	1/3	0/1
	7		0/2	NT	0/3	0/3	0/1
	8		0/3	NT	2/3	0/3	0/1
	9		0/3	NT	3/3	1/3	0/1
	10		0/3	NT	0/3	1/3	1/1
	11		0/3	NT	1/3	1/3	0/1
	12		0/3	NT	0/3	0/3	1/1
	13		0/3	NT	0/3	1/3	1/1
	14		1/3	NT	0/3	1/3	1/1
	15		0/3	NT	0/3	0/3	0/1
WPI XMRV/P-MLV Subjects		†			†		
	1		0/1	0/2	0/2	1/2	0/1
	2		0/1	0/1	1/2	0/2	0/1
	3		0/1	0/1	1/2	0/2	0/1
	4		0/2	0/2	2/2*	0/3	1/1
	5		0/1	1/2	0/2	0/2	1/1
	6		0/1	1/2	0/2	0/2	0/1
	7		0/2	0/2	0/2	0/2	0/1
	8		0/1	1/2	1/2	1/2	0/1
	9		0/1	0/3	1/2	0/2	0/1
	10		0/1	0/2	1/2	1/2	1/1
Lo et al. XMRV/P-MLV Subjects		†			†		
	1		0/3	0/2	1/2	0/2	0/1
	2		0/3	0/2	1/2	0/2	0/1
	3		0/3	0/2	1/2	1/2	0/1
	4		0/3	0/2	1/2	1/2	0/1
	5		0/3	0/1	2/2	0/2	0/1
Spiked Controls		‡			‡		
	1		3/5	NT	NT	NT	5/5

*NT, not tested.
†The kappa for the serology for the negative controls between NCI/Ruscetti and WPI is 0.21.
‡The kappa for the serology for the WPI XMRV/P-MLV subjects between NCI/Ruscetti and WPI is −0.20.
§The kappa for the serology for the Lo et al. XMRV/P-MLV subjects between NCI/Ruscetti and WPI is 0.00.
□The kappa for the serology for all cohort subjects between NCI/Ruscetti and WPI is −0.08.

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No. 17

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
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一般的名称	人全血液		研究報告の公表状況	Homaira N, Rahman M, Hossain MJ, Epstein JH, Sultana R, Khan MS, Podder G, Nahar K, Ahmed B, Gurley ES, Daszak P, Lipkin WI, Rollin PE, Comer JA, Ksiazek TG, Luby SP. Epidemiol Infect. 2010 Nov;138(11):1630-6. Epub 2010 Apr 12.	公表国 バングラデシュ	
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研究報告の概要

○2007年バングラデシュにおけるニパウイルスのヒトーヒト感染の発生
2007年2月、バングラデシュ北西部でニパウイルス(NiV)脳炎のアウトブレイクがあり、7人が感染、そのうち3人が死亡した。一連の患者は、発症中の当該患者と身体的接触後7日～14日後に発症した。症例群をコントロール群と比べると、患者と同室に滞在すること(100%vs.9.5%、P<0.001)や、身体的接触がある(83%vs.0%、P<0.001)方がより発症しやすかった。当該患者の感染原因は明らかになっていないが、アウトブレイク1カ月後の調査で、その地域付近のオオコウモリのうち50%がNiV抗体を持っていたことから、この地域でのウイルスの存在が確認出来る。看病する家族は、潜在的感染源である体液からNiVに感染しないように、予防(石鹸で手を洗う、身体的接触を避ける)を習慣付けることが重要である。

使用上の注意記載状況・その他参考事項等

人全血液-LR「日赤」
照射人全血液-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
バングラデシュにおいて、ニパウイルスのヒトからヒトへの伝播が示唆されたとの報告である。	日本赤十字社では、輸血感染症対策として受付時に海外渡航歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、発熱などの体調不良者を献血不適としている。今後も引き続き、新興・再興感染症の発生状況等に関する情報の収集に努める。

12

MedDRA/J Ver.14.1J

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Nipah virus outbreak with person-to-person transmission in a district of Bangladesh, 2007

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SUMMARY

In February 2007 an outbreak of Nipah virus (NiV) encephalitis in Thakurgaon District of northwest Bangladesh affected seven people, three of whom died. All subsequent cases developed illness 7–14 days after close physical contact with the index case while he was ill. Cases were more likely than controls to have been in the same room (100% *vs.* 9·5%, OR undefined, $P<0·001$) and to have touched him (83% *vs.* 0%, OR undefined, $P<0·001$). Although the source of infection for the index case was not identified, 50% of *Pteropus* bats sampled from near the outbreak area 1 month after the outbreak had antibodies to NiV confirming the presence of the virus in the area. The outbreak was spread by person-to-person transmission. Risk of NiV infection in family caregivers highlights the need for infection control practices to limit transmission of potentially infectious body secretions.

Key words: Bangladesh, Nipah virus, person-to-person transmission.

INTRODUCTION

In Bangladesh, Nipah virus (NiV) was first identified as the cause of an outbreak of encephalitis in 2001 in Meherpur District [1, 2]. Four additional outbreaks were identified between 2001 and 2005 [1–4]. Antibodies reactive to NiV antigen have been detected in pteropid bats in both India and Bangladesh [1, 5].

Person-to-person transmission of NiV infection, following human infection directly from the environment, was noted in previous outbreaks in the Indian subcontinent. In a NiV outbreak in Siliguri, India in 2001, 45 patients (75%) had a history of hospital exposure to other patients with NiV infection [6]. In Faridpur District, Bangladesh in 2004 NiV case-patients in Faridpur were seven times more likely than non-patients to have had close contact with one of the NiV patients [odds ratio (OR) 6·7, 95% confidence interval (CI) 2·9–16·8, $P<0·001$] [2].

On 9 February 2007, a physician at Rangpur Medical College Hospital, one of 10 hospitals involved in active NiV encephalitis surveillance in Bangladesh, reported a cluster of fatal encephalitis involving a husband and a wife residing in the Haripur Upazila (subdistrict) of Thakurgaon District. Both patients had similar symptoms and died within an interval of 2 weeks. A collaborative team including the Institute of Epidemiology Disease Control and Research (IEDCR) and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), began an investigation on 10 February 2007. The objectives of the investigation were to identify the cause of the outbreak and the risk factors for developing illness.

METHODS

Case definition and identification

We defined suspected case-patients as persons having fever with altered mental status or new onset of seizures (severe illness), or persons having fever with headache or cough (mild illness), residing in the outbreak area with an onset of illness between 15 January and 28 February 2007. The team visited the outbreak village and asked the community health workers and community residents if they were aware of any patient who was suffering from fever with seizure or altered mental status, or who had died from these symptoms in their neighbourhood. We also asked them about case-patients suffering from fever with headache and/or cough. The team then visited the local hospital in order to identify suspected case-patients. Team members also investigated all the deaths in the outbreak village between January and February. We obtained a history of illness and general information about exposures for each suspected case-patient. We asked the local health authority of the affected subdistrict to report to the IEDCR if they identified any further suspected case-patient having fever and altered mental status or seizures who sought treatment in the local subdistrict health complex during February.

The team collected blood samples from living suspected case-patients, which were centrifuged in the field and transported on wet ice to IEDCR, where they were stored at −70°. Samples were tested with an immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) that detects IgM antibodies specific for NiV antigens [7].

We defined a confirmed case of NiV infection as a suspected case-patient with detectable IgM to NiV. The team defined a probable NiV case-patient as a patient with fever and altered mental status who lived in the same village as a confirmed case-patient during the outbreak period, but from whom serum was not available because the patient died before a specimen could be collected.

Qualitative study

A team of experienced anthropologists conducted in-depth interviews and informal discussions with available confirmed and probable case-patients, their family members and friends, and other residents in these communities with the goals of exploring potential exposures to NiV and identifying appropriate proxy respondents for deceased cases or cases that were too sick to interview. The anthropologists also collected information about symptoms of the disease, caregiving practices and health facility utilization by persons affected by the outbreak.

Case-control study

We conducted a case-control study to investigate exposures associated with NiV infection, including person-to-person transmission. Probable and confirmed case-patients were enrolled as cases. We selected three controls for each case-patient. Controls were selected starting from the fourth closest house to the case-patient where no members were ill during the outbreak. The household resident closest in age to the case-patient was eligible to participate as a control. Participation was voluntary. If the selected household resident declined to participate, a resident from the next closest house was asked to participate.

The qualitative team selected proxy respondents for each case-patient who had died or was unable to respond. The proxy respondents included family members and friends of the case-patients who were most knowledgeable about their activities and probable risk exposures in the preceding 1 month before illness. Multiple proxy respondents were common. The investigation team used a standardized questionnaire to collect information on demographics, symptoms of illness, and possible risk factors associated with NiV transmission including history of consumption of date palm juice prior to illness, exposure

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to animals and exposure to ill patients, including touching, staying in the same room, feeding, sharing a bed or cleaning body secretions of a NiV patient.

Bat survey

A team of veterinarians from ICDDR,B with assistance from the Consortium for Conservation Medicine located two bat roosts which were 1 km and 15 km distant from the outbreak village. Bats were captured using mist nets and were anaesthetized during sample collection and released at the point of capture after sampling from 24 February to 9 March 2007. All the captured bats from which blood samples were collected were *P. giganteus*.

All bat blood samples were kept on ice until the end of each day when serum was separated and stored in liquid nitrogen. At the end of each day, blood samples were transferred to liquid nitrogen and transported to ICDDR,B where they were stored at −70 °C and then shipped on dry ice to the Australian Animal Health Laboratory for laboratory diagnosis. All the blood samples were assayed for antibodies against NiV using a serum neutralization test.

Statistics

We analysed socio-demographic and clinical profiles of the case-patients using descriptive statistics. For the case-control study, we used ORs to estimate the association of each exposure with disease and calculated 95% CIs around the ORs. We used the χ^2 test when expected cell sizes were >5 and Fisher's exact test when expected cell sizes were <5 and considered association to be statistically significant if the P value was <0·05. We used an unmatched analysis because neighbours were chosen as controls to ensure that controls and case-patients were representative of the same population and not to control for confounding factors. Because the primary hypothesis was that the index case was the source of NiV transmission for the subsequent cases, we excluded the index case, but none of the controls in the analyses of person-to-person transmission.

Ethics

All human study participants gave informed consent for participation in this investigation. The Ethical Review Committee at ICDDR,B reviewed and approved a protocol for encephalitis surveillance and outbreak investigation. Bat capture and sample collection was conducted under a protocol approved by the Institutional Animal Care and Use Committee.

RESULTS

Descriptive epidemiology

Eleven serum samples were collected from 13 suspected case-patients. Five suspected case-patients had IgM antibodies against NiV by capture ELISA and were thus confirmed cases. Two suspected case-patients had fever and altered mental status, but died before samples could be collected and were categorized as probable cases. These two probable cases were the index case and his wife. The remainder of the analysis was performed on these seven confirmed or probable case-patients. Five of these case-patients (three confirmed and two probable) had fever with altered mental status and three (60%) of them died. A total of five case-patients, including the two probable cases, were hospitalized. The mean age of case-patients was 24 years (range 19–30 years) and five (71%) were male. The median duration from onset of fever to death was 5·6 days (range 5–7) (Table 1). Fever (100%), altered consciousness (71%) along with vomiting (71%) and cough (71%) were the most common symptoms (Table 1).

Qualitative findings

The index case first developed fever on 21 January 2007 which progressed to headache, cough, breathing difficulties, convulsions, loss of consciousness and finally death 5 days later. In total 14 people who were family members, relatives or friends had physical contact with the index case when he was ill; six (43%) of them developed NiV infection. Five of the subsequent cases had contact with the index case only during the last 2 days of his illness (incubation period 7–14 days). The dates of illness onset for subsequent cases ranged from 2 to 8 February 2007 (Fig. 1). None of the caregivers of the subsequent cases developed illness.

During the first 4 days of illness the index case was cared for at home, primarily by his wife and sister-in-law. They fed him, cleaned him and wiped froth and saliva from his mouth. They also massaged oil on his head and body to relieve him of pain. His wife shared the same bed with him and provided care throughout his illness. She became severely ill 14 days after the husband's illness began and died within 6 days of illness.

A day before his death, the index case developed a severe cough and breathing difficulty. He was taken to a local doctor accompanied by a friend and a cousin. A chest radiograph of the index case taken during this period showed diffuse bilateral opacity in both lung fields suggesting features of acute respiratory distress syndrome (Fig. 2). The friend became ill 11 days later and died after 7 days. The cousin also became ill 14 days after his physical contact with the index case.

The day after his chest radiograph, the index case developed reduced level of consciousness, and was admitted to a hospital where he died on the same day. The driver of a micro bus who helped transport and carry him to the hospital developed NiV infection 10 days after exposure.

While the index case was hospitalized, his wife's sister and one of his friends visited him in the hospital

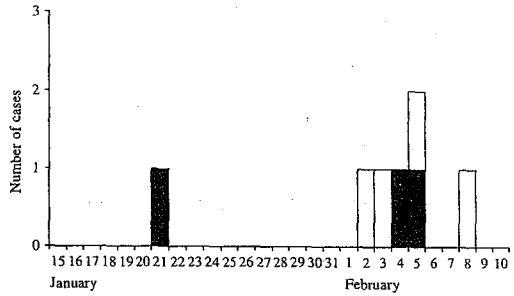

Fig. 1. Distribution of NiV cases by date of onset of illness, Haripur Upazila (subdistrict), Thakurgaon District, Bangladesh, January–February 2007. □, Alive; ■, died.

Table 1. *Characteristics of case-patients, Haripur Upazila, Thakurgaon District, Bangladesh, February 2007*

Characteristics	$n=7$ (%)
Age	
Mean (years)	24
Median (range)	24 (19–30)
Male	5 (71)
Occupation	
Student	2 (29)
Housewife	1 (14)
Businessman	2 (29)
Driver	2 (29)
Clinical feature	
Fever	7 (100)
Severe fatigue/weakness	6 (86)
Headache	3 (43)
Vomiting	5 (71)
Cough	5 (71)
Respiratory distress	4 (57)
Altered mental status	5 (71)
Muscle pain	4 (57)
Restlessness	4 (57)
Unconscious	2 (29)
Joint pain	1 (14)
Case fatality	3 (43)
Onset of illness to death ($n=3$), mean (range)	5·6 (5–7)

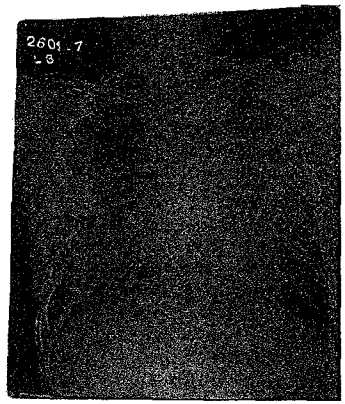

Fig. 2. Chest X-ray of the index case showing features of acute respiratory distress syndrome.

Table 2. *Bivariate analysis of risk factors for Nipah virus infection, Haripur Upazila, Thakurgaon District, Bangladesh, February 2007*

Risk factors	No. (%) of cases with this risk factor	No. (%) of controls with this risk factor	OR	95% CI	*P* value
Male sex	4 (67%)	8 (38%)	3·25	0·48–22	0·2
Climbed trees	1 (17)	4 (19%)	0·85	0·07–9·4	1·00
Physical contact with living animal					
Pig	0 (0%)	0 (%)	Undefined		
Fruit bat	0 (0%)	0 (0%)	Undefined		
Cow	4 (67%)	13 (62%)	1·23	0·182–8·33	1·00
Goat	2 (33%)	9 (43%)	0·67	0·099–4·5	1·00
Ate any animal that had been sick	0 (0%)	1 (5%)	Undefined		1·00
Drank raw date palm sap	1 (17%)	0 (0%)	Undefined		0·22
Visited the index case in a hospital	6 (100%)	0 (0%)	Undefined		<0·001
Touched the index case when he was sick	5 (83·3%)	0 (0%)	Undefined		<0·001
Been present in the same room with the index case when he was sick	6 (100%)	2 (9·5%)	Undefined		<0·001
Been present in the same room with the index case when he was coughing	6 (100%)	0 (0%)	Undefined		0·04

OR, Odds ratio; CI, confidence interval.

and fed and touched him. Both of them developed NiV infection within 10 days of contact.

Case-control study

We used proxy interviews for the three dead case-patients, but not for any controls. The mean age for cases and controls was similar [mean age (±S.D.) 24±4 years in cases *vs.* 24±7 years in controls, $t=-0{\cdot}097$, $P=0{\cdot}9$]. Cases were more likely to be males than controls but this could be due to chance (67% males in case-patient group *vs.* 38% in control group, OR 3·2, 95% CI 0·5–22, $P=0{\cdot}2$).

NiV case-patients were more likely than controls to have consumed raw date palm sap in the 15 days prior to illness (29% in case-patients *vs.* 0% in controls, OR undefined, $P=0{\cdot}056$). Two (29%) of the case-patients including the index case who had consumed raw date palm juice bought it from a vendor in the local village market. Although there were sick goats in the outbreak-affected community, none of the cases or controls had any contact with them or any other sick animal within 15 days prior to illness. Moreover, there was no report of contact with fruit bats.

In the analysis for person-to-person transmission, case-patients were more likely than controls to have been present in the same room with (100% *vs.* 9·5%, OR undefined, $P=0{\cdot}000$) or touched (83% *vs.* 0%, OR undefined, $P=0{\cdot}000$) the index case. In a sub-analysis in those who stayed in the same room with the index case, case-patients were more likely than controls to be present in the same room when he was coughing (100% *vs.* 0%, OR undefined, $P=0{\cdot}04$). Only case-patients had hospital exposure to other NiV case-patients (86% *vs.* 0%, $P=0{\cdot}000$), with all reporting visits to the index case in the hospital (Table 2).

Bat study

The team captured and sampled 118 *P. giganteus* bats; 29 of which were juvenile bats. Of the 67 bats sampled 1 km from the outbreak village, 34 (51%) tested positive on serum neutralization test [median titre 30, range 5 to >640]. Three of the 34 serum neutralization test-positive bats had NiV antibody titres >640. In the neighbouring village, 15 km away, 27/51 bats (53%) had serum neutralizing antibodies to NiV [median titre 20, range 5–320]. Of the 61 pteropid bats that were seropositive seven were juvenile bats [median titre 15, range 5–20].

DISCUSSION

Several lines of evidence suggest person-to-person transmission as the primary route of transmission in this outbreak. The epidemic curve showing a gap of

12–18 days between the single primary case and the secondary cases corresponds with the incubation period of human NiV infection [8]. Many of the index case's contacts (43%), who came in physical contact with the index case when he was ill, subsequently became ill. In the case-control study, case-patients were significantly more likely to be in contact with the index case and were significantly more likely to be near him when he was coughing. As subsequent cases were limited to close contacts of the primary case, and none of the contacts of the subsequent cases developed illness, we conclude that the index case was the only NiV transmitter in this outbreak.

Five (83%) of the subsequent cases came in contact with the index case only during the last 2 days of his illness when he developed respiratory symptoms. NiV has been isolated from human saliva, urine, nasal and pharyngeal secretions [9, 10] and there is evidence of spread of NiV infection from direct contact with respiratory secretions or other body secretions of infected pigs and humans [2, 11, 12]. The probability of NiV transmission is probably amplified during the last stages of illness when respiratory symptoms become more prominent and perhaps the concentration of virus in respiratory secretions increases. In Bangladesh, as the level of physical contact with the patient intensifies with the severity of the disease [13], this further increases the risk of transmission.

The NiV neutralizing antibody prevalence was >50% in the bats sampled from the outbreak area which suggests that NiV has circulated in this population of bats. The result is consistent with findings in other pteropid bat populations in Malaysia, India, and Bangladesh [1, 5, 14, 15]. The bat survey was performed approximately 1 month after the onset of illness in the index case, and it is possible that infected bats were present in the colony around the time of the first human infection. Furthermore, the index case had no evidence of exposure to clinically ill domestic animals. He also had history of drinking raw date palm juice before his illness which has been associated with NiV infection in a previous outbreak investigation [3]. These lines of evidence suggest that the virus was probably transmitted directly from its natural reservoir, rather than an intermediate domestic animal.

A limitation of our study is its reliance on proxy interviews for some of the case-patients. This may have obscured some exposure information. However, we started our investigation within 14 days of the death of the index case, and collected information from several proxy respondents thus reducing the likelihood that we failed to collect information on probable exposure to risk factors. Another limitation is the lack of serological data from controls. There is evidence of subclinical infection of NiV from Malaysia [16] which could have reduced power to identify association due to erroneous inclusion of cases as controls. Even with this potential limitation our results identified a biologically credible pathway for transmission.

Findings from outbreaks in Siliguri and Faridpur illustrate that human-to-human transmission has occurred repeatedly in the Indian subcontinent. The social norm in Bangladesh is that family members and loved ones provide hands-on care to sick patients [13]. Further, hospital healthcare workers in Bangladesh are reluctant to provide hands-on care to admitted patients which increases the risk of transmission to family members and relatives who provide care without any training or supplies to reduce the risk of transmission [13]. Efforts to educate caregivers of their risk especially at later stages of illness, while maintaining sensitivity to cultural mores, and promoting basic infection control practices such as washing hands with soap after handling patients and avoiding close physical contact [2] could limit transmission of NiV and other diseases in people who care for sick patients.

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