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
				2007年12月25日	該当なし		
一般的名称	別紙のとおり		研究報告の公表状況	CDC／Travelers' Health, Outbreak Notice. January 8, 2008		公表国 ウガンダ	
販売名（企業名）	別紙のとおり						

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
問題点：2007年後半にウガンダで発生したエボラ出血熱のアウトブレイクは、既知の4つのエボラウイルス株と異なる新たなウイルス株が原因である可能性がある。 米国CDCとウガンダ保健省は、ウガンダ西部に位置するBundibugyo地区におけるエボラ出血熱のアウトブレイクについて報告した。アウトブレイクは早ければ2007年8月から始まった可能性がある。2008年1月3日までに148人が罹患し、37人が死亡した。症例サンプルの遺伝子解析により、既知の4つのエボラウイルス株と異なる、新たなウイルス株であることが示された。しかし、確定するには更なる解析が必要である。	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。

MedDRA10.1

⑨

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乾燥ペプシン処理人免疫グロブリン、⑥乾燥スルホ化人免疫グロブリン、⑦乾燥スルホ化人免疫グロブリン*、⑧乾燥濃縮人活性化プロテインC、⑨乾燥濃縮人血液凝固第Ⅷ因子、⑩乾燥濃縮人血液凝固第Ⅸ因子、⑪乾燥抗破傷風人免疫グロブリン、⑫抗 HBs 人免疫グロブリン、⑬トロンビン、⑭フィブリノゲン加第XⅢ因子、⑮乾燥濃縮人アンチトロンビンⅢ、⑯ヒスタミン加人免疫グロブリン製剤、⑰人血清アルブミン*、⑱人血清アルブミン*、⑲乾燥ペプシン処理人免役グロブリン*、⑳乾燥人血液凝固第Ⅸ因子複合体*、㉑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献血静注グロブリン "化血研"、⑥献血ベニロン－Ｉ、⑦ベニロン*、⑧注射用アナクトC2,500 単位、⑨コンファクトＦ、⑩ノバクトＭ、⑪テタノセーラ、⑫ヘパトセーラ、⑬トロンビン "化血研"、⑭ボルヒール、⑮アンスロビンＰ、⑯ヒスタグロビン、⑰アルブミン 20%化血研*、⑱アルブミン 5%化血研*、⑲静注グロブリン*、⑳ノバクトＦ*、㉑アンスロビンＰ1500 注射用
報告企業の意見	エボラ出血熱はエボラウイルスによる急性熱性疾患であり、ラッサ熱、マールブルグ病、クリミア・コンゴ出血熱とともに、ウイルス性出血熱の一疾患である。エボラウイルスは、フィロウイルス科（*Filoviridae*）に属し、1 本鎖 RNA を核酸として持ち、エンベロープを有する。短径が 80～100nm、長径が 700～1,500nm で、多形性（U 字状、ひも状、ぜんまい状等）を示す。 エボラ出血熱は、現在までアフリカの中央部でのみ発生している。感染者・患者の血液や体液との接触によりヒトからヒトへ感染が拡大し、多数の死者を出す流行を起こす。ヒトは終末宿主であるが、動物、昆虫などの自然宿主、媒介動物については全く不明である。そのため、自然界からヒトへの感染経路も不明である。 発症は突発的で進行も早い。潜伏期は 2～21 日で、汚染注射器を通した感染では早く、接触感染では長い。発熱、頭痛、腹痛、咽頭痛、筋肉痛、胸部痛及び出血等の症状がみられ、重篤化する。致死率は患者の 53～88%と高い。 弊所の血漿分画製剤の製造工程には、冷エタノール分画工程、ウイルス除去膜ろ過工程あるいは加熱工程等の原理の異なるウイルス除去及び不活化工程が存在しているので、ウイルスクリアランスが期待される。 各製造工程のウイルス除去・不活化効果は、「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）」に従い、ウシウイルス性下痢ウイルス（BVDV）、仮性狂犬病ウイルス（PRV）、ブタパルボウイルス（PPV）、A 型肝炎ウイルス（HAV）または脳心筋炎ウイルス（EMCV）をモデルウイルスとして、ウイルスプロセスバリデーションを実施し、評価を行っている。今回報告したエボラウイルスは、エンベロープの有無、核酸の種類等からモデルウイルスとしては BVDV が該当すると考えられるが、上記バリデーションの結果から、BVDV の除去・不活化効果を有することを確認している。 また、これまでに当該製剤によるエボラウイルス感染の報告例は無い。 以上の点から、当該製剤はエボラウイルス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

Outbreak Notice
Updated: Ebola Outbreak in the District of Bundibugyo, Uganda
This information is current as of today, January 24, 2008 at 20:11

Updated: January 08, 2008

The U.S. CDC and the Ministry of Health of Uganda have reported an Ebola hemorrhagic fever outbreak in the Bundibugyo district located in the Western part of the country. The outbreak may have begun as early as August 2007. As of January 3, 2008, 148 people have become ill and 37 people have died. Genetic analysis of samples from case-patients indicated that this is a new virus strain distinct from the four known strains of Ebola virus. However, further studies will be needed before this can be verified.

Ebola hemorrhagic fever is a rare, serious viral disease which develops suddenly, with common symptoms of fever, headache, joint and muscle aches, sore throat, and weakness. Diarrhea, vomiting, and stomach pain start after the first symptoms. A skin rash may develop. By the third or fourth day of illness some people with Ebola hemorrhagic fever may develop internal and external bleeding, shock and organ failure.

Ebola is spread through direct contact with blood or other body fluids (e.g., saliva, urine) of infected persons or objects that have been contaminated with infected body fluids. People who have close contact with a nonhuman primate infected with the virus are also at risk.

Recommendations for U.S. Travelers

The World Health Organization (WHO) has reported that there is no need for any travel restrictions to Uganda. Generally, the risk of contracting Ebola virus is low for travelers. CDC recommends that anyone traveling to Uganda take the following steps to prevent Ebola virus infection:

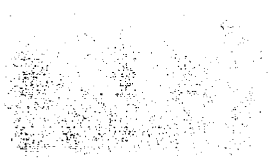
- Avoid contact with Ebola patients and their body fluids.
- Avoid touching used needles or other medical waste.
- Avoid contact with wild animals and bushmeat, including primates.

More Information

For information about the current situation, see the WHO report at www.who.int.

For additional information on Ebola hemorrhagic fever, please see http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/ebola.htm.

To learn more about traveling to areas with hemorrhagic fevers, see the Viral Hemorrhagic Fevers section of *CDC Health Information for International Travel 2008.*



医薬品 研究報告 調査報告書

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2007. 10. 22</td><td>新医薬品等の区分
該当なし</td><td rowspan="3">機構処理欄</td></tr>
<tr><td>一般的名称</td><td colspan="2">人赤血球濃厚液</td><td rowspan="2">研究報告の公表状況</td><td rowspan="2">山田正仁, 篠原もえ子, 野崎一朗, 浜口毅, 中村好一, 北本哲之, 佐藤猛, 水澤英洋, CJDサーベイランス委員会. 2007年プリオン研究会</td><td>公表国</td></tr>
<tr><td>販売名(企業名)</td><td colspan="2">赤血球濃厚液-LR「日赤」(日本赤十字社)
照射赤血球濃厚液-LR「日赤」(日本赤十字社)</td><td>日本</td></tr>
<tr><td>研究報告の概要</td><td colspan="5">○わが国におけるヒトのプリオン病の実態:最近のサーベイランスデータ
我が国の人口動態統計では、クロイツフェルト・ヤコブ病(CJD)による死亡は過去20年以上に渡り右肩上がりに増加傾向を示し、2005年は人口100万対1.23人であった。『プリオン病および遅発性ウイルス感染症に関する調査研究班』・CJDサーベイランス委員会による現行のプリオン病調査は1999年から始まった。そこでは、プリオン病が疑われる全患者について、画像、脳脊髄液マーカー、プリオン蛋白(PrP)遺伝子型、病理などの検査を含めた実地調査を行うことを原則としている。このシステムにより過去8年間に918例がプリオン病と判定された。病型別では、孤発性CJD716例(78.0%)、遺伝性プリオン病128例(14.0%)、感染性(獲得性)CJD72例(7.8%)[変異型CJD(vCJD)1例/硬膜移植後CJD(dCJD)71例]、および分類不能2例(0.2%)であった。PrP遺伝子に変異がないことを確認した孤発性CJD387例の臨床像をみると、進行が速く(無動性無言まで9ヵ月未満)特徴的な脳波所見を有する典型例は74%、それ以外の非典型例が26%を占めた。進行が遅く特徴的脳波を欠く、最も非典型的な群は、他群と較べて脳脊髄液マーカーやMRI上の高信号の陽性率も低く、Parchi分類でMM2型に属し、特に視床型が臨床診断上問題であった。遺伝性プリオン病128例の分類では、コドン180変異42例(32.9%)、コドン200変異CJD26例(20.4%)、コドン102変異25例(19.6%)、コドン232変異17例(13.3%)他の順であり、欧米ではほとんどないコドン180、232変異が多くみられるなどの特色があった。dCJDは1996年の佐藤班による全国調査以来、硬膜移植歴が判明したものを合計すると129例になった。dCJDの中で、比較的緩徐な進行を示し特徴的脳波を欠き脳にPrP斑を認める非典型例(プラーク型)の割合は剖検例では48%であり、臨床例を含めるとdCJD全体の約1/3を占めると考えられた。2007年7月現在、vCJDは英国短期滞在歴がある1例のみである。</td><td>使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-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</td></tr>
<tr><td colspan="3">報告企業の意見</td><td colspan="3">今後の対応</td><td rowspan="2"></td></tr>
<tr><td colspan="3">CJDサーベイランス委員会による調査では過去8年間に日本国内で918例がプリオン病と判定された。また、人口動態統計ではCJDによる死亡者数は過去20年以上に亘って増加傾向を示し、2005年は人口100万対1.23人であったとの報告である。</td><td colspan="3">日本赤十字社は、vCJDの血液を介する感染防止の目的から、献血時に過去の海外渡航歴(旅行及び居住)を確認し、欧州36ヶ国に一定期間滞在したドナーを無期限に献血延期としている。また、英国滞在歴を有するvCJD患者が国内で発生したことから、平成17年6月1日より1980～96年に1日以上の英国滞在歴のある方からの献血を制限している。今後もCJD等プリオン病に関する新たな知見及び情報の収集に努める。</td></tr>
</table>

Poster-20

わが国におけるヒトのプリオン病の実態：最近のサーベイランスデータ

山田正仁[1,6]、篠原もえ子[1]、野崎一朗[1]、浜口　毅[1]、中村好一[2,6]、北本哲之[3,6]、佐藤　猛[4,6]、水澤英洋[5,6]、CJDサーベイランス委員会[6]

[1]金沢大学大学院　脳老化・神経病態学（神経内科）、[2]自治医大公衆衛生、[3]東北大学大学院プリオン蛋白研究部門、[4]東大和病院、[5]東京医科歯科大学大学院脳神経病態学（神経内科）、[6]厚生労働省・難治性疾患克服研究事業「プリオン病及び遅発性ウイルス感染症に関する調査研究班」・CJDサーベイランス委員会

わが国の人口動態統計では、クロイツフェルト・ヤコブ病（CJD）による死亡は過去20年以上に渡り右肩上がりに増加傾向を示し、2005年は人口100万対1.23人であった。『プリオン病および遅発性ウイルス感染症に関する調査研究班』・CJDサーベイランス委員会による現行のプリオン病調査は1999年から始まった。そこでは、プリオン病が疑われる全患者について、画像、脳脊髄液マーカー、プリオン蛋白（PrP）遺伝子型、病理などの検査を含めた実地調査を行うことを原則としている。このシステムにより過去8年間に918例がプリオン病と判定された。病型別では、孤発性CJD 716例（78.0%）、遺伝性プリオン病128例（14.0%）、感染性（獲得性）CJD 72例（7.8%）[変異型CJD（vCJD）1例／硬膜移植後CJD（dCJD）　71例]、および分類不能2例（0.2%）であった。PrP遺伝子に変異がないことを確認した孤発性CJD 387例の臨床像をみると、進行が速く（無動性無言まで9ヶ月未満）特徴的な脳波所見を有する典型例は74%、それ以外の非典型例が26%を占めた。進行が遅く特徴的脳波を欠く、最も非典型的な群は、他群と較べて脳脊髄液マーカーやMRI上の高信号の陽性率も低く、Parchi分類でMM2型に属し、特に視床型が臨床診断上問題であった（Hamaguchi *et al. Neurology* 64:643, 2005)。遺伝性プリオン病128例の分類では、コドン180変異 42例（32.9%）、コドン200変異CJD 26例（20.4%）、コドン102変異25例（19.6%）、コドン232変異 17例（13.3%）他の順であり、欧米ではほとんどないコドン180、232変異が多くみられるなどの特色があった。dCJDは1996年の佐藤班による全国調査以来、硬膜移植歴が判明したものを合計すると129例になった。dCJDの中で、比較的緩徐な進行を示し特徴的脳波を欠き脳にPrP斑を認める非典型例（プラーク型）の割合は剖検例では48%であり、臨床例を含めるとdCJD全体の約1/3を占めると考えられた（Noguchi-Shinohara *et al. Neurology* 69:360, 2007)。2007年7月現在、vCJDは英国短期滞在歴がある1例（Yamada *et al. Lancet* 367:874, 2006）のみである。

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 2007年12月17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	PloS Pathogens. 2007;3:1895-1906	公表国 インド洋南西地域およびインド	
販売名(企業名)	別紙のとおり					

研究報告の概要

問題点：2005年から2006年にかけてのレユニオン諸島でのチクングニヤウイルス感染のアウトブレイクは、ヒトスジシマカをチクングニヤウイルスのベクターとし、また致命的な感染が報告された流行であるという特徴を持っていた。

以前の流行では、チクングニヤウイルス（CHIKV）感染は非致死性の感染症と考えられていた。しかし、レユニオン諸島でアウトブレイクしたCHIKV感染は、266,000人が発症し、260人の死者が出た。CHIKVは、*Aedes aegypti*（ネッタイシマカ）をプライマリーベクターとするが、2005～2006年のレユニオン諸島でのアウトブレイクにおけるベクターは*Aedes albopictus*（ヒトスジシマカ）であった。
研究者らは、CHIKVのエンベロープ蛋白(E1)の226番目のアミノ酸がアラニンからバリンに変異していることを明らかにした。この変異により、CHIKVはネッタイシマカと比較して、ヒトスジシマカへの感染性が増し、その唾液腺でより早く増殖するようになり、また乳のみマウスへもより効率的に感染するようになった。
一つのアミノ酸置換がベクターの特異性に影響を与えるという今回の結果は、通常のベクターが存在しない地域で変異ウイルスが流行を起こした理由をうまく説明をしている。これは、ウイルスが新しい地域に入り込んだときにどのように感染サイクルを確立するかに関する重要な仮説となる。ヒトスジシマカは広く分布しているため、この変異はCHIKVの分布が欧州やアメリカ大陸に拡がる可能性を増大させることとなる。

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。

MedDRA10.1

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乾燥ペプシン処理人免疫グロブリン、⑥乾燥スルホ化人免疫グロブリン、⑦乾燥スルホ化人免疫グロブリン*、⑧乾燥濃縮人活性化プロテインC、⑨乾燥濃縮人血液凝固第Ⅷ因子、⑩乾燥濃縮人血液凝固第Ⅸ因子、⑪乾燥抗破傷風人免疫グロブリン、⑫抗 HBs 人免疫グロブリン、⑬トロンビン、⑭フィブリノゲン加第XⅢ因子、⑮乾燥濃縮人アンチトロンビンⅢ、⑯ヒスタミン加人免疫グロブリン製剤、⑰人血清アルブミン*、⑱人血清アルブミン*、⑲乾燥ペプシン処理人免役グロブリン*、⑳乾燥人血液凝固第Ⅸ因子複合体*、㉑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献血静注グロブリン "化血研"、⑥献血ベニロン-Ⅰ、⑦ベニロン*、⑧注射用アナクトC2,500 単位、⑨コンファクトF、⑩ノバクトM、⑪テタノセーラ、⑫ヘパトセーラ、⑬トロンビン "化血研"、⑭ボルヒール、⑮アンスロビンP、⑯ヒスタグロビン、⑰アルブミン20%化血研*、⑱アルブミン 5%化血研*、⑲静注グロブリン*、⑳ノバクトF*、㉑アンスロビンP1500 注射用
報告企業の意見	チクングニヤウイルス (Chikungunya virus) は、トガウイルス科 (*Togaviridae*) のアルファウイルス属 (*Alphavirus*) に分類される1本鎖の RNA を核酸として持つ直径 70nm のエンベロープを有する球状粒子である。いままでに日本国内での感染・流行はないが、2006 年 12 月に海外からの輸入症例 2 例が報告された。チクングニヤウイルスは蚊によって媒介されるが、感染後ウイルス血症を起こすことから、血液を介してウイルス感染する可能性を完全に否定できないため本報告を行った。 弊所の血漿分画製剤の製造工程には、冷エタノール分画工程、ウイルス除去膜ろ過工程あるいは加熱工程等の原理の異なるウイルス除去・不活化工程が存在しているので、仮にウイルスが原料血漿に混入していたとしても、ウイルスクリアランスが期待される。各製造工程のウイルス除去・不活化効果は、「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)」に従い、ウシウイルス性下痢ウイルス (BVDV)、仮性狂犬病ウイルス (PRV)、ブタパルボウイルス (PPV)、A 型肝炎ウイルス (HAV) または脳心筋炎ウイルス (EMCV) をモデルウイルスとして、ウイルスプロセスバリデーションを実施し、評価を行っている。今回報告したチクングニヤウイルスはエンベロープの有無、核酸の種類等からモデルウイルスとしては BVDV が該当すると考えられるが、上記バリデーションの結果から、BVDV の除去・不活化効果を有することを確認している。 また、これまでに弊所の血漿分画製剤によるチクングニヤウイルス感染の報告例は無い。 以上の点から、当該製剤はチクングニヤウイルス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

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A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential

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Chikungunya virus (CHIKV) is an emerging arbovirus associated with several recent large-scale epidemics. The 2005–2006 epidemic on Reunion island that resulted in approximately 266,000 human cases was associated with a strain of CHIKV with a mutation in the envelope protein gene (E1-A226V). To test the hypothesis that this mutation in the epidemic CHIKV (strain LR2006 OPY1) might influence fitness for different vector species, viral infectivity, dissemination, and transmission of CHIKV were compared in *Aedes albopictus*, the species implicated in the epidemic, and the recognized vector *Ae. aegypti*. Using viral infectious clones of the Reunion strain and a West African strain of CHIKV, into which either the E1-226 A or V mutation was engineered, we demonstrated that the E1-A226V mutation was directly responsible for a significant increase in CHIKV infectivity for *Ae. albopictus*, and led to more efficient viral dissemination into mosquito secondary organs and transmission to suckling mice. This mutation caused a marginal decrease in CHIKV *Ae. aegypti* midgut infectivity, had no effect on viral dissemination, and was associated with a slight increase in transmission by *Ae. aegypti* to suckling mice in competition experiments. The effect of the E1-A226V mutation on cholesterol dependence of CHIKV was also analyzed, revealing an association between cholesterol dependence and increased fitness of CHIKV in *Ae. albopictus*. Our observation that a single amino acid substitution can influence vector specificity provides a plausible explanation of how this mutant virus caused an epidemic in a region lacking the typical vector. This has important implications with respect to how viruses may establish a transmission cycle when introduced into a new area. Due to the widespread distribution of *Ae. albopictus*, this mutation increases the potential for CHIKV to permanently extend its range into Europe and the Americas.

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Introduction

The large-scale epidemic of the mosquito-transmitted alphavirus, Chikungunya virus (CHIKV), began in Kenya in 2004 and spread to several Indian Ocean islands including the Comoros, Mauritius, the Seychelles, Madagascar, Mayotte and Reunion. On Reunion island alone there were approximately 266,000 cases (34% of the total island population) [1–6]. In the continuing Indian epidemic there have been at least 1.4M cases reported [7–10] with continued expansion in Sri Lanka and Indonesia. CHIKV had not been reported to cause fatalities in prior outbreaks; however, during the outbreak on Reunion island, CHIKV was associated with at least 260 deaths [11,12]. The strain of CHIKV responsible for the Indian Ocean island epidemic has been well-characterized in cell culture and mosquito models [13–15]; however, the underlying genetic basis of the atypical phenotype of this CHIKV strain remains unknown.

CHIKV is transmitted by *Aedes* species mosquitoes, primarily *Ae. aegypti*. However, the 2005–2006 CHIKV epidemic on Reunion island was unusual because the vector responsible for transmission between humans was apparently the Asian tiger mosquito, *Ae. albopictus* [3,16]. This conclusion is based on several factors. This species is known to be susceptible to CHIKV infection and although infectious virus was not isolated from *Ae. albopictus* during the epidemic, CHIKV RNA was detected (X. de Lamballerie, personal communication). Furthermore, the species is anthropophylic, was abundant during the epidemic, and other potential vectors specifically *Ae. aegypti* were relatively scarce with a very limited distribution (P. Reiter, personal communication). *Ae. albopictus* is abundant and widely distributed in urban areas of Europe and the United States of America [17–22]. CHIKV infections have been reported in many travelers returning to the US and Europe [12,23–26] causing concern that the virus could be introduced and become established in these areas [1,27,28]. In August and September of 2007, a CHIKV–*Ae. albopictus* transmission cycle was reported for the first time in Europe, with an estimated 254 human cases occurring in Italy [29,30].

Alphaviruses are enveloped single stranded positive sense RNA viruses. Genomic RNA, of ≈ 12,000 nt, encodes four non-structural (ns1–4) and three main structural proteins (capsid, E2 and E1). At neutral pH, E2 and E1 exist as heterodimers in which E2 forms spikes on the virion surface that interact with cellular receptors. The E1 protein lies below E2 and mediates fusion of the viral and cellular membranes during viral entry [31].

Analysis of CHIKV genome microevolution during the 2005–2006 Indian Ocean epidemic identified an alanine to valine mutation at position 226 in the E1 envelope glycoprotein (E1-A226V) among viral isolates obtained during the

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outbreak [32]. The reason for this was unclear but it was hypothesized that the E1-A226V mutation might influence infectivity of CHIKV for mosquito vectors [11,32]. Interestingly, earlier studies have identified that a P→S mutation in the same position of the E1 glycoprotein is responsible for the modulation of Semliki Forest virus's (SFV, a member of the alphavirus family) requirements for cholesterol in the target membrane [33]. It also has been shown that the presence of this mutation results in more efficient growth of SFV in *Ae. albopictus* mosquitoes [34]. However, no evidence has been presented to directly correlate the release from the cholesterol dependence, associated with the E1-P226S mutation in SFV, with a growth advantage in *Ae. albopictus*. It is unknown if dependence on cholesterol for growth in mosquito cells is a requirement of all alphaviruses.

To test the hypothesis that the E1-A226V mutation might influence the fitness of CHIKV in mosquito vectors, we compared the effect of this mutation on CHIKV mosquito infectivity, the ability to disseminate into heads and salivary glands, and the relative fitness in competition assays for transmission by *Ae. albopictus* and *Ae. aegypti* to suckling mice. We also analyzed the effect of the E1-A226V mutation on CHIKV cholesterol dependence for growth in mosquito C6/36 (*Ae. albopictus*) cells. Here we report findings that a single nucleotide change, which arose during the epidemic, significantly increases fitness of the virus for *Ae. albopictus* mosquitoes and was associated with CHIKV dependence on cholesterol in the mosquito cell membrane. This change likely enhanced CHIKV transmission by an atypical vector and contributed to the maintenance and scale of the epidemic.

Results

Effect of E1 A226V Mutation on Fitness of CHIKV in *Ae. albopictus* Mosquitoes

To test the hypothesis that the E1-A226V mutation altered CHIKV infectivity for *Ae. albopictus* mosquitoes, CHIKV infectious clones derived from an epidemic Reunion island human isolate were used [15], including one clone (LR-GFP-226V) expressing enhanced green fluorescent protein (eGFP). Clones were further engineered to express E1 protein containing an alanine at position E1-226 (LR-GFP-226A) representing the CHIKV genotype prevalent prior to the outbreak gaining momentum (Figure S1). RNAs produced from both clones (LR-GFP-226V and LR-GFP-226A) have comparable specific infectivity values, produced similar viral titers following transfection into BHK-21 cells (Table S1) and have similar growth kinetics in mosquito (C6/36) and mammalian (BHK-21) cells lines (Figure S2A and S2B).

The relative infectivity of LR-GFP-226V and LR-GFP-226A viruses was analyzed in female *Ae. albopictus* mosquitoes orally exposed to serial 10-fold dilutions of CHIKV (LR-GFP-226 V or A). To determine whether infection rates correlate with blood meal titer, midguts dissected from mosquitoes at 7 days post-infection (dpi) were analyzed for foci of eGFP-expressing cells by fluorescence microscopy (Figure 1A; Table 1). In two independent experiments, LR-GFP-226V virus was found to be approximately 100-fold more infectious to *Ae. albopictus* than LR-GFP-226A virus ($p<0.01$). To test if the infectivity phenotype was directly linked to the mutation, the complementary reverse mutation, E1-A226V, was introduced into an infectious clone of a West African CHIKV strain, 37997-GFP (37997-GFP-226A) (Figure S1). The Reunion and 37997 strains of CHIKV are distantly related, with only 85% nucleotide sequence identity. The parental 37997-GFP-226A and the 37997-GFP-226V viruses were indistinguishable in cell culture experiments (Table S1; Figure S2C and S2D); however, *in vivo* experiments in *Ae. albopictus* mosquitoes revealed that the E1-A226V mutation significantly decreases the oral infectious dose 50 (OID_{50}) value for the 37997-GFP-226V virus ($p<0.01$) to an extent similar to that observed for LR-GFP-226V virus (Figure 1B; Table 1). These data conclusively demonstrate that the single E1-A226V point mutation is therefore sufficient to significantly reduce the OID_{50} of the 37997-GFP virus ($p<0.01$) in *Ae. albopictus* mosquitoes equivalent to that observed for the LR-GFP-226V virus (Figure 1A; Table 1).

To further evaluate viral fitness of the epidemic CHIKV E1-A226V mutation in *Ae. albopictus*, viral competition experiments were performed. Although our CHIKV eGFP-expressing infectious clones, have similar infection properties in mosquitoes as wild-type viruses [15,35], to address potential concerns that eGFP expression might influence OID_{50} values, we constructed LR-226A and LR-ApaI-226V viruses without eGFP and employed them in viral competition experiments (Figures 2A and S1). LR-ApaI-226V was derived from previously described CHIK-LR ic, by the introduction of a silent marker mutation, A6454C, in order to add an *Apa*I restriction site into the coding sequence. It was shown that the A6454C mutation does not affect the specific infectivity value (Table S1), the viral titer after RNA transfection into BHK-21 cells value (Table S1), the viral growth kinetics in BHK-21 and C6/36 cells (Figure S3), infectivity for and viral titers in *Ae. aegypti* and *Ae. albopictus* mosquitoes (Table S2), or viral fitness for growth in BHK-21 and C6/36 cells as determined by competition assay (Figure S4). These data indicate that the introduced mutation is indeed silent and does not affect the fitness of LR-ApaI-226V.

For viral competition experiments LR-ApaI-226V virus (10^7 plaque-forming units (pfu)) was mixed with an equal

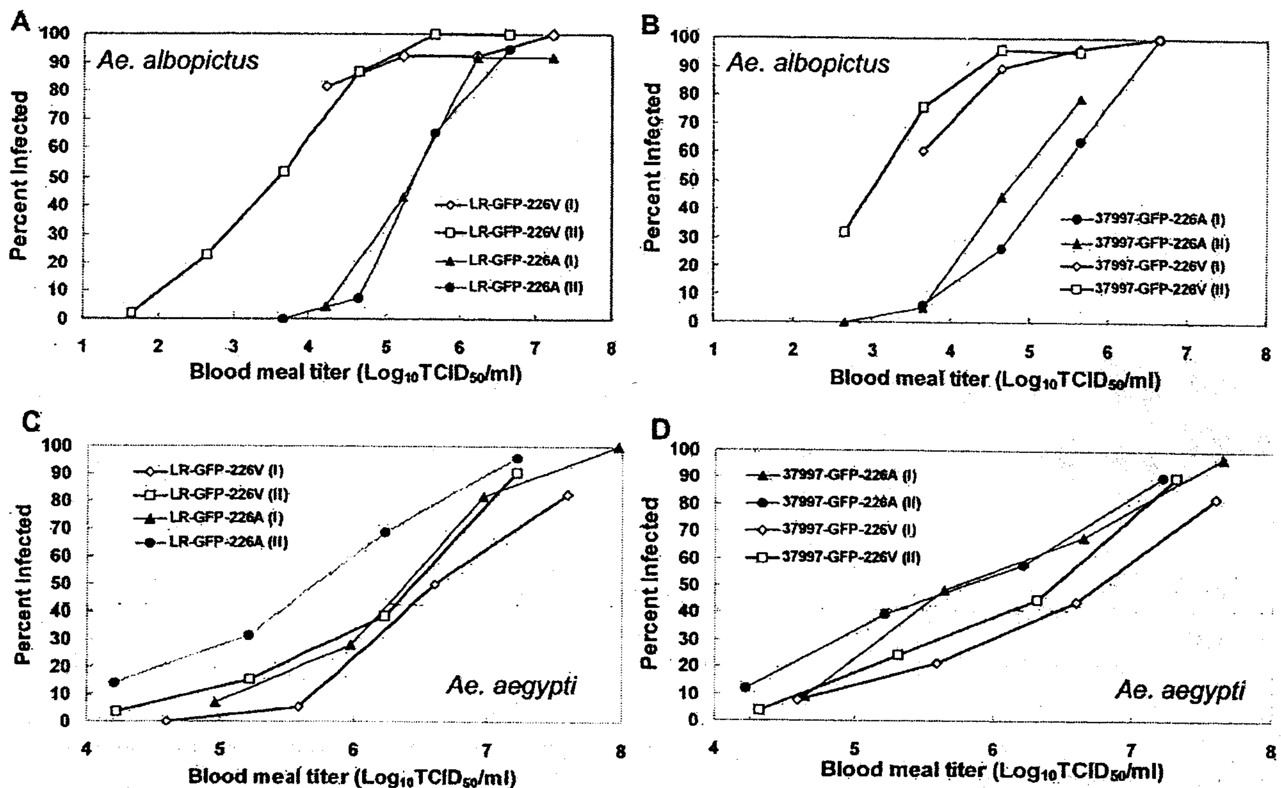

Figure 1. Effect of E1-A226V Mutation on CHIKV-GFP Viruses *Ae. albopictus* and *Ae. aegypti* Midgut Infectivity
Percent of orally infected *Ae. albopictus* (A, B) and *Ae. aegypti* (C, D) mosquitoes presented with blood meals containing various concentration of eGFP-expressing CHIK viruses. Serial 10-fold dilutions of viruses in the backbone of Reunion (LR-GFP-226V and LR-GFP-226A) (A, C) and 37997 (37997-GFP-226A and 37997-GFP-226V) (B, D) strains of CHIKV were made in L-15 medium followed by mixing the samples with defibrinated sheep blood. Mosquitoes were dissected at 7 dpi and eGFP expression in infected midguts was analyzed by fluorescence microscopy. A mosquito was considered infected if at least one foci of eGFP-expressing cells was present in the midgut. The experiments were performed twice for each virus (I and II).
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amount of LR-226A virus. LR-ApaI-226V and LR-226A viruses are indistinguishable in cell culture experiments (Figure S3). Mixtures of LR-ApaI-226V and LR-226A viruses were orally presented to *Ae. albopictus* mosquitoes in a blood meal, and midguts were examined at 7 dpi. The relative amount of RNA derived from LR-ApaI-226V in the midgut cells increased 5.7±0.6 times as compared to the initial relative amount of LR-ApaI-226V RNA in the blood meal sample (Figure 2B). These data support our observation that the E1-A226V mutation enhances infectivity of CHIKV for *Ae. albopictus* mosquitoes and furthermore demonstrate that the mutation could provide an evolutionary advantage over E1-226A viruses in an atypical vector and may have perpetuated the outbreak in a region where *Ae. albopictus* was the predominant anthropophilic mosquito species.

To determine if the enhanced midgut infectivity associated with the E1-A226V mutation may result in more efficient viral dissemination into secondary tissues, the kinetics of viral dissemination by LR-GFP-226V and LR-GFP-226A into salivary glands, and competition between LR-ApaI-226V and LR-226A for dissemination into mosquito heads were analyzed (Figure 3A and 3B). LR-GFP-226V virus disseminated more rapidly into *Ae. albopictus* salivary glands at all time points, with a significant difference at 7 dpi (p=0.044, Fisher's exact test). Similarly, in three of four replicates of competition experiments, RNA from LR-ApaI-226V virus was dramatically more abundant in the heads of *Ae. albopictus* mosquitoes as compared to RNA from LR-226A (Figure 3B, lines 1, 3, 4), although in one replica LR-ApaI-226V RNA was only slightly more abundant as compared to the initial viral RNA ratio (Figure 3B, line 2). This variability of the results may be due to random pooling of mosquito heads. Thus, replicate two may have included more heads negative for LR-

Table 1. $Log_{10}OID_{50}$/ml for CHIKV in *Ae. albopictus* Mosquitoes

Backbone	Exp[a]	Virus	Mosquitoes Analyzed[b]	Log_{10} $OID_{50}\pm CI_{95}$[c]	p Value
CHIK Reunion	1	LR-GFP-226V	98	<4.22	p<0.01
		LR-GFP-226A	101	5.42±0.29	
	2	LR-GFP-226V	171	3.52±0.28	p<0.01
		LR-GFP-226A	93	5.48±0.23	
CHIK 37997	1	37997-GFP-226A	131	5.20±0.22	p<0.01
		37997-GFP-226V	138	3.31+0.42	
	2	37997-GFP-226A	129	4.90±0.25	p<0.01
		37997-GFP-226V	136	3.06±0.32	

OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63).
[a]Experiment number.
[b]Number of mosquitoes used to estimate $Log_{10}OID_{50}$/ml.
[c]95% confidence intervals.
doi:10.1371/journal.ppat.0030201.t001

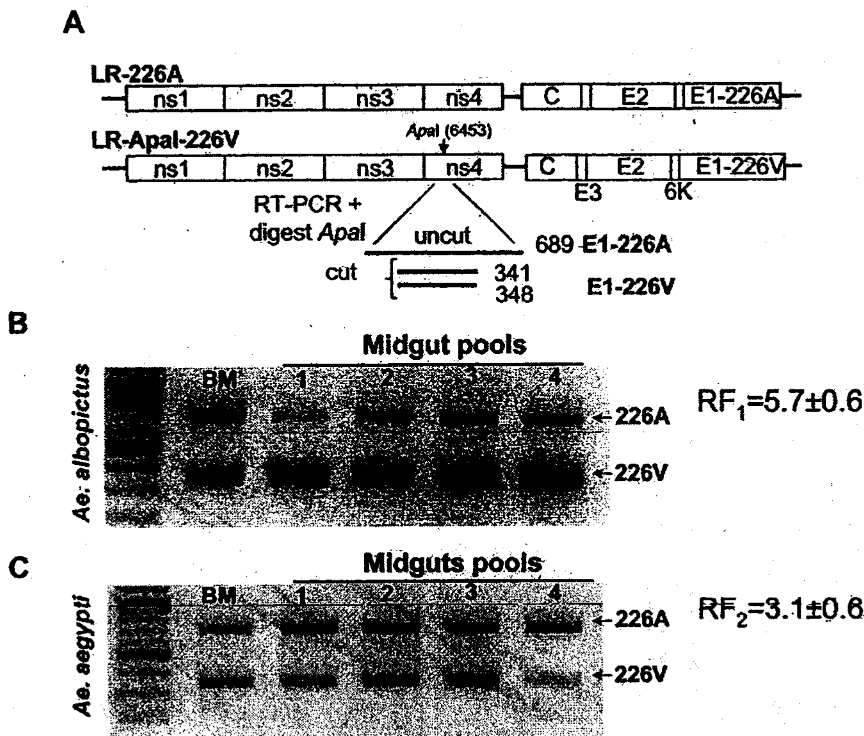

Figure 2. Schematic Representation of Competition Experiments (A) and Competition between LR-ApaI-226V and LR-226A Viruses for Colonization of Midgut cells of *Ae. albopictus* (B) and *Ae. aegypti* (C) Mosquitoes

10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus* (B) and *Ae. aegypti* (C). Viral RNAs were extracted from four pools of eight to ten midguts at 7 dpi. RT-PCR products were digested with *ApaI*, separated in 2% agarose gel, and gels were stained using ethidium bromide.

BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1–4 ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the eight to ten midguts per replica.

Relative fitness (RF_1) of LR-Apa-226V to LR-226A was calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio between 226V and 226A in the blood meal.

Relative fitness (RF_2) of LR-226A to LR-Apa-226V was calculated as a ratio between 226A and 226V bands in the sample, divided to the control ratio between 226A and 226V in the blood meal.

Results expressed as the average of four replicas ± standard deviation (SD).

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ApaI-226V relative to heads positive for LR-226A RNA. Another possibility is that at some point during viral dissemination from the midguts into mosquito heads, LR-226A may replicate more rapidly than LR-ApaI-226V. To further investigate this relationship, *Ae. albopictus* mosquitoes were orally presented with either LR-ApaI-226V or LR-226A and whole mosquito body viral titers were compared at different time points pi. Surprisingly, no significant differences between viral titers were found, with the exception of 1 dpi, where the LR-ApaI-226V titer was 0.5 Log_{10} tissues culture infectious dose 50 percent end point titer (Log_{10} $TCID_{50}$/mosquito) higher than of the LR-226A titer (Figure 4A). This may be due to more efficient colonization of *Ae. albopictus* midguts by LR-ApaI-226V. The absence of significant differences in viral titers at later time points may be due to variation in viral titers among individual mosquitoes. Competition between LR-ApaI-226V and LR-226A was analyzed at different time points in order to investigate the relationship between replication of LR-ApaI-226V and LR-226A viruses in *Ae. albopictus* mosquitoes (Figure 4B). As expected, the viral RNA from LR-ApaI-226V was predominant at the early time points of 1 and 3 dpi. Interestingly, between 3 and 5 dpi the viral RNA ratio shifted toward LR-226A virus indicating that at these time points, LR-226A replicates more efficiently in some mosquito tissues (Figure 4B). This short period of time may have a slight effect on the overall outcome of competition for dissemination into salivary glands because there is a reverse shift in the RNA ratio between days 5 and 7 toward LR-ApaI-226V virus, which continues through 14 dpi. These data indicate that the E1-A226V mutation not only increases midgut infectivity but also is associated with more efficient viral dissemination from the midgut into secondary organs, suggesting that the E1-A226V mutation would increase transmissibility of CHIKV by *Ae. albopictus* mosquitoes.

A competition assay between LR-ApaI-226V and LR-226A viruses was used to examine transmission by *Ae. albopictus* to suckling mice to assess the potential for the E1-A226V mutation to influence virus transmission. *Ae. albopictus* mosquitoes were orally presented with a mixture of LR-ApaI-226V and LR-226A viruses and at 14 dpi were allowed to feed on suckling mice. Mice were sacrificed and bled on day 3 following exposure and the presence of CHIKV RNA in the blood was analyzed by RT-PCR followed by restriction digestion with *ApaI* (Figure 5B). Blood obtained from 100% of experimental mice contained detectible amounts of viral

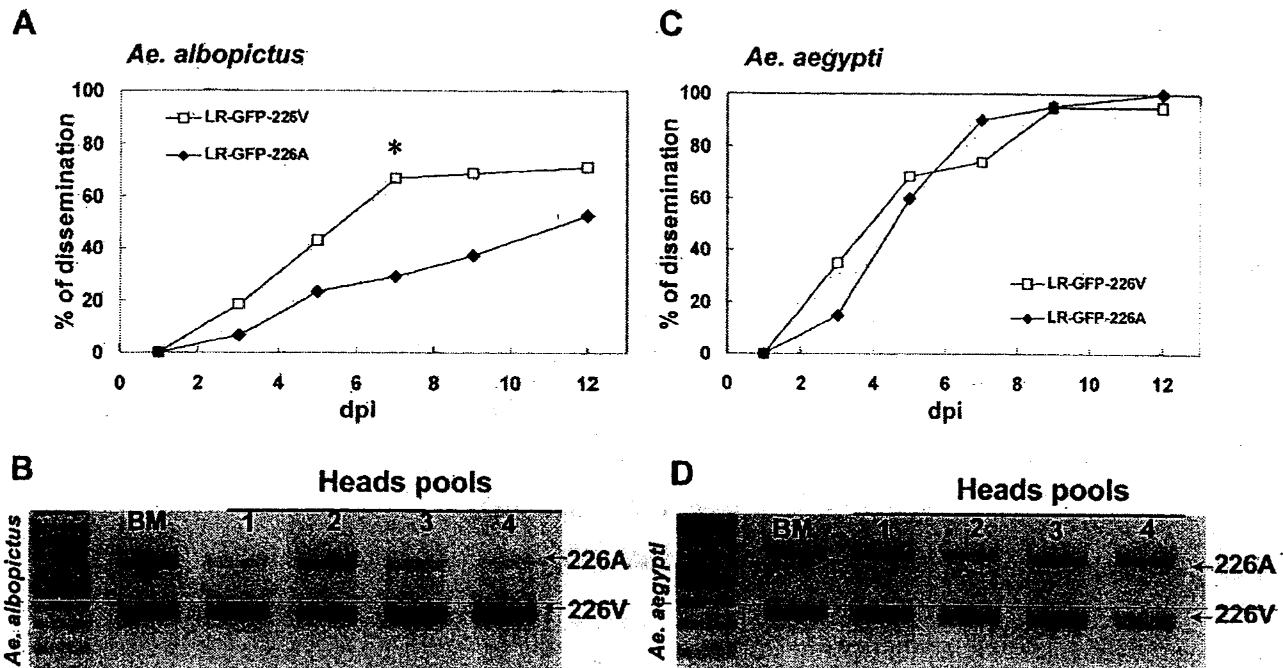

Figure 3. Effect of E1-A226V Mutation on CHIKV Dissemination into Salivary Glands and Heads of *Ae. albopictus* and *Ae. aegypti* Mosquitoes

Ae. albopictus (A) and *Ae. aegypti* (C) mosquitoes were orally infected with LR-GFP-226V and LR-GFP-226A. At the indicated time points, 16–21 mosquitoes were dissected and salivary glands were analyzed for eGFP expression. Percent of dissemination was estimated as a ratio of the number of mosquitoes with eGFP-positive salivary glands to the number of mosquitoes with eGFP-positive midguts. For *Ae. albopictus*, infectious blood meal titers were 5.95 and 6.52 $Log_{10}TCID_{50}$/ml for LR-GFP-226V and LR-GFP-226A, respectively. For *Ae. aegypti*, the infectious blood meal titer was 6.95 $Log_{10}TCID_{50}$/ml for both LR-GFP-226V and LR-GFP-226A viruses. Dissemination rates were compared statistically by Fisher's exact test using SPSS version 11.5. Asterisk indicates $p < 0.05$.

(B and D) Competition between LR-ApaI-226V and LR-226A for dissemination into heads of *Ae. albopictus* and *Ae. aegypti* mosquitoes. 10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus* (B) and *Ae. aegypti* (D). Viral RNAs were extracted from four pools of five heads collected at 12 dpi. RT-PCR products were digested with *ApaI*, separated in 2% agarose gel, and gels were stained using ethidium bromide. BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples. 1–4 ratio of LR-ApaI-226V and LR-226A RNA in four independent replicas of the five pooled heads per replica.

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RNA, indicating that virus was transmitted by *Ae. albopictus* mosquitoes to suckling mice. More importantly, in all six mice analyzed, RNA derived from LR-ApaI-226V was the predominant viral RNA species, indicating that under the conditions of competition for transmission, the E1-A226V mutation directly increases CHIKV transmission by *Ae. albopictus* mosquitoes. Interestingly, in the control experiment in which mice were subcutaneously inoculated with ≈ 50 pfu of 1:1 mixture of LR-ApaI-226V and LR-226A viruses, RNAs from both viruses were readily detected and no difference was observed in the viral RNA ratio 3 dpi (Figure 5A) indicating that at least in mice, E1-A226V is not associated with changes in viral fitness.

Effect of E1 A226V Mutation on Fitness of CHIKV in *Ae. aegypti* Mosquitoes

Since the E1-A226V mutation confers a fitness advantage in *Ae. albopictus*, it is unknown why this mutation had not been observed previously. It is possible that this change might have a deleterious effect on viral fitness in the vertebrate host, although our data of direct competition of LR-ApaI-226V and LR-226A viruses in suckling mice (Figure 5A) and analysis of CHIKV cellular tropism of four clinical isolates from Reunion (which have either A or V at position E1-226) [14], suggest that this is unlikely. An alternative hypothesis is that the E1-A226V mutation might compromise the fitness of CHIKV or have neutral fitness effects in the mosquito species which served as a vector for CHIKV prior to its emergence on Reunion island. Since *Ae. aegypti* has generally been regarded as the main vector for CHIKV prior to the emergence on Reunion island, we analyzed the effect of the E1-A226V mutation on fitness of CHIKV in *Ae. aegypti*.

In contrast to the results obtained in *Ae. albopictus* mosquitoes, OID_{50} values of viruses containing the E1-226V in the backbone of the Reunion and 37997 strains of CHIKV were approximately 0.5 $Log_{10}OID_{50}$/ml higher than the OID_{50} values of E1-226A viruses in all experiments using *Ae. aegypti*. These differences were statistically significant for one out of two replicates for each virus pair (Figure 1C and 1D; Table 2). A competition assay examining LR-ApaI-226V and LR-226A virus infection in *Ae. aegypti* midguts, demonstrated that LR-226A virus out-competed LR-ApaI-226V virus at 7 dpi in all four replicates using ten midguts per replicate and that the amount of LR-226A RNA increased on average 3.1 times as compared to the initial blood meal RNA ratio (Figure 2C). These data suggest that the E1-A226V mutation has a slight negative effect on CHIKV infectivity of *Ae. aegypti* midguts.

The effect of the E1-A226V mutation on the ability of CHIKV to disseminate into *Ae. aegypti* secondary organs was also analyzed (Figure 3C and 3D). LR-GFP-226V and LR-GFP-

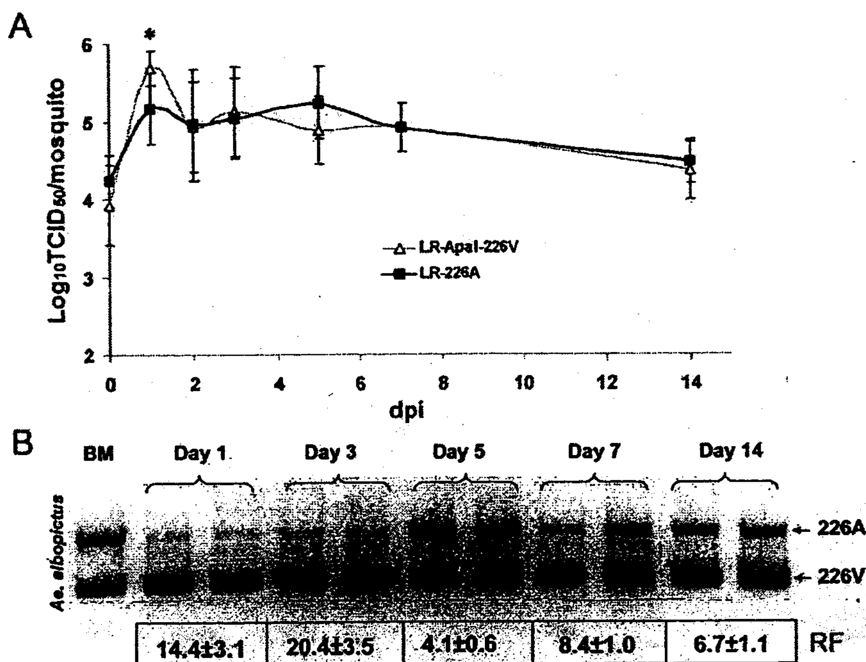

Figure 4. Effect of E1-A226V Mutation on CHIKV Kinetics of Viral Growth in Bodies of *Ae. albopictus* Mosquitoes

(A) Virus production in orally infected *Ae. albopictus* mosquitoes. Infected mosquitoes were sampled at 0, 1, 2, 3, 5, 7, and 14 dpi and titrated on Vero cells to estimate average titer ± standard deviation of eight whole mosquitoes. Differences in viral titers were analyzed by pairwise t-tests. Asterisk indicates $p < 0.05$.

(B) Kinetics of competition between LR-ApaI-226V and LR-226A in bodies of *Ae. albopictus* mosquitoes. 10^7 pfu of LR-ApaI-226V and LR-226A were mixed and orally presented to *Ae. albopictus*. Infected mosquitoes were sampled at 1, 3, 5, 7, and 14 dpi. For each time point, viral RNA was extracted from two pools of ten mosquitoes.

BM - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples.

RF - relative fitness of LR-Apa-226V to LR-226A was calculated as a ratio between 226V and 226A bands in the sample, divided to the control ratio between 226V and 226A in the blood meal. Results expressed as average of two replicas ± standard deviation.

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226A viruses both have similar kinetics of dissemination into salivary glands following oral infection using titers 1–2 $Log_{10}TCID_{50}$ higher than their OID_{50} value in *Ae. aegypti* (Figure 3C). In a competition assay, both LR-ApaI-226V and LR-226A viruses disseminated similarly into the heads of *Ae. aegypti*. In two of four replicas, there was a slight increase in the relative amount of LR- 226A RNA (Figure 3D, lines 1, 4); whereas the other two replicas showed a decrease in LR-226A RNA (Figure 2D, lines 2, 3), relative to the initial ratio of the RNA of LR-ApaI-226V and LR-226A viruses in the blood meal. A competition of LR-ApaI-226V and LR-226A viruses for transmission by *Ae. aegypti* to suckling mice was also analyzed (Figure 5C). In contrast to transmission by *Ae. albopictus* mosquitoes, five out of six mice fed upon by *Ae. aegypti* contained comparable amounts of RNA derived from both viruses and only one out of six mice contained RNA derived exclusively from LR-ApaI-226V.

E1-A226V Mutation Modulates Cholesterol Dependence of CHIKV

It has been previously shown that a P→S mutation in the same E1-226 position of SFV releases cholesterol dependence of the virus in C6/36 cells [33] and results in significantly more rapid growth of SFV in *Ae. albopictus* mosquitoes after intrathoracic inoculation [34]. To determine if a requirement for cholesterol in the cell membrane is important for CHIKV, we analyzed cholesterol dependence of CHIKV E1-226A and E1-226V viruses (Figure 6). Growth curves of E1-226A and E1-226V viruses in the background of Indian Ocean and West African strains of CHIKV were almost indistinguishable when grown in C6/36 cells maintained in L-15 supplied with standard 10% FBS (Figure 6A). However, when the cells were depleted of cholesterol, LR-226A and 37997-226A viruses replicated significantly more rapidly than LR-226V and 37997-226V viruses, reaching 3 $Log_{10}TCID_{50}$/ml higher titer at 1, 2 and 3 dpi (Figure 6B). These data indicate that adaptation of CHIKV to *Ae. albopictus* mosquitoes coincides with CHIKV dependence on cholesterol in the target cell membrane.

Discussion

The CHIKV outbreak in Reunion is unique because it is the first well-documented report of an alphavirus outbreak for which *Ae. albopictus* was the main vector. Interestingly, this was also the first Chikungunya epidemic during which fatal infections were reported. Our data clearly indicate that an E1-A226V mutation in CHIKV results in increased fitness of CHIKV in *Ae. albopictus* mosquitoes with respect to midgut infectivity, dissemination to the salivary glands, and transmission to a vertebrate species. These data demonstrate that a single E1-A226V mutation is sufficient to dramatically increase the ability of different strains of CHIKV to infect *Ae. albopictus* mosquitoes and that this substitution requires no

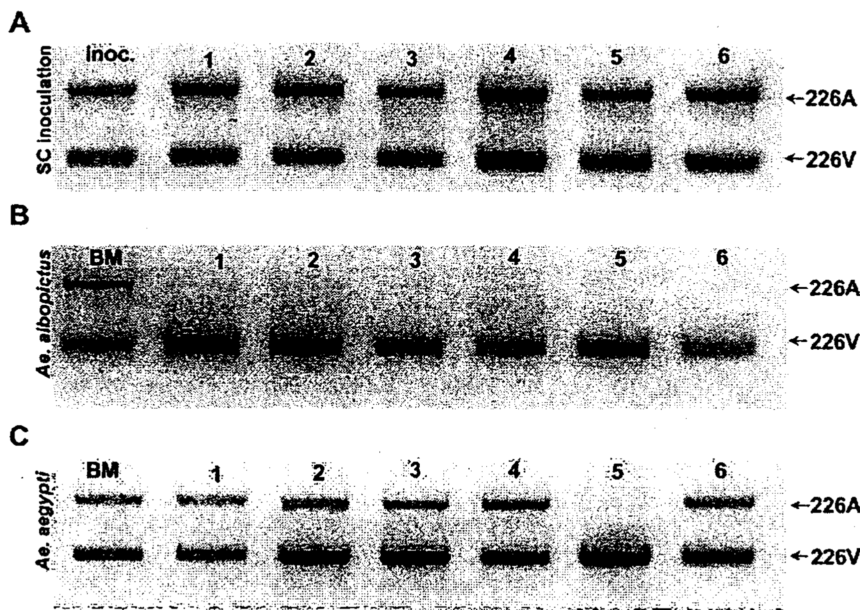

Figure 5. Effect of E1-A226V Mutation on CHIKV Transmission by *Ae. albopictus* and *Ae. aegypti* Mosquitoes

(A) Six 2- to 3-day-old suckling mice (Swiss Webster) were subcutaneously infected with a 20-μl mixture of ~ 25 pfu LR-Apa-226V and ~ 25 pfu of LR-226A viruses.

(B and C) *Ae. aegypti* and *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 pfu/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. At 13 dpi, ten to 15 mosquitoes were placed in separate paper cartons and starved for 24 h. The next day, the mosquitoes in each carton were presented with a 2- to 3-day-old suckling mouse (Swiss Webster).

Mice were returned to their cage and sacrificed on day 3 post-exposure. Blood from each individual mouse (~ 50 μl) was collected and immediately mixed with 450 μl of TRIzol reagent for RNA extraction.

BM and inoc. - initial ratio of LR-ApaI-226V and LR-226A in blood meal samples and inoculum for subcutaneous infection. 1–6 ratio of LR-ApaI-226V and LR-226A RNA in six individual mice.

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additional adaptive mutations to gain intermolecular compatibility. These complimentary experimental data demonstrate that a single mutation is sufficient to modify viral infectivity for a specific vector species and as a consequence, can fuel an epidemic in a region that lacks the typical vector. These observations provide the basis for an explanation of the observed rapid shift among CHIKV genotypes to viruses containing the E1-A226V mutation during the Reunion outbreak [32].

Interestingly, our data and data from previous studies [36,37] indicate that prior to acquiring the E1-A226V mutation, CHIKV is capable of producing high enough viremia in humans to efficiently infect *Ae. albopictus* mosquitoes. One explanation of the evolutionary force which allowed CHIKV to be selected so rapidly into a CHIKV strain which is adapted to *Ae. albopictus*, is that the increased infectivity (lower OID_{50}) of CHIKV E1-A226V mutants for *Ae. albopictus* means that the human viremic thresholds required for *Ae. albopictus* infection would likely occur earlier and be sustained for longer. Several recent studies indicate that during the course of human viremia, which last up to 6 days, CHIKV loads can reach up to $3.3x10^9$ RNA copies per ml of the blood [38,39], which corresponds to 6–7 $Log_{10}TCID_{50}$/ml [39]. Earlier studies that utilized a suckling mouse brain titration protocol, which is more sensitive than titration on Vero cells, also found that human viremia often exceeded 6 $Log_{10}SMICLD_{50}$/0.02 ml [40]. Based on viremia studies in rhesus monkeys that can develop up to 7.5 Log/ml if assayed by suckling mice brain titration [41] and a maximum viremia of only 5.5 Log_{10}/ml based on Vero cell titration [42], we believe that viremias in humans would correlate to 6–7 $Log_{10}TCID_{50}$/ml. From these data we calculate that the maximum virus load which can be achieved in human blood is 1–2 $Log_{10}TCID_{50}$/ml higher than the $Log_{10}OID_{50}$/ml for E1-226A viruses but 3–4 $Log_{10}TCID_{50}$/ml higher than the $Log_{10}OID_{50}$/ml for E1-226V viruses. During the course of viremia there should therefore be a substantial time frame in which CHIKV blood load is high enough for E1-226V viruses to infect *Ae. albopictus* but below the threshold for infection

Table 2. $Log_{10}OID_{50}$/ml for CHIKV in *Ae. aegypti* Mosquitoes

Backbone	Exp[a]	Virus	Mosquitoes Analyzed[b]	Log_{10} $OID_{50}\pm CI_{95}$[c]	p Value
CHIK Reunion	1	LR-GFP-226V	65	6.77±0.40	p<0.1
		LR-GFP-226A	103	6.12±0.28	
	2	LR-GFP-226V	107	6.26±0.30	p<0.05
		LR-GFP-226A	53	5.62±0.33	
CHIK 37997	1	37997-GFP-226A	161	5.77±0.25	p<0.01
		37997-GFP-226V	162	6.59±0.34	
	2	37997-GFP-226A	136	5.83±0.30	p<0.1
		37997-GFP-226V	127	6.34±0.29	

OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63).
[a]Experiment number.
[b]Number of mosquitoes used to estimate $Log_{10}OID_{50}$/ml.
[c]95% confidence intervals.
doi:10.1371/journal.ppat.0030201.t002

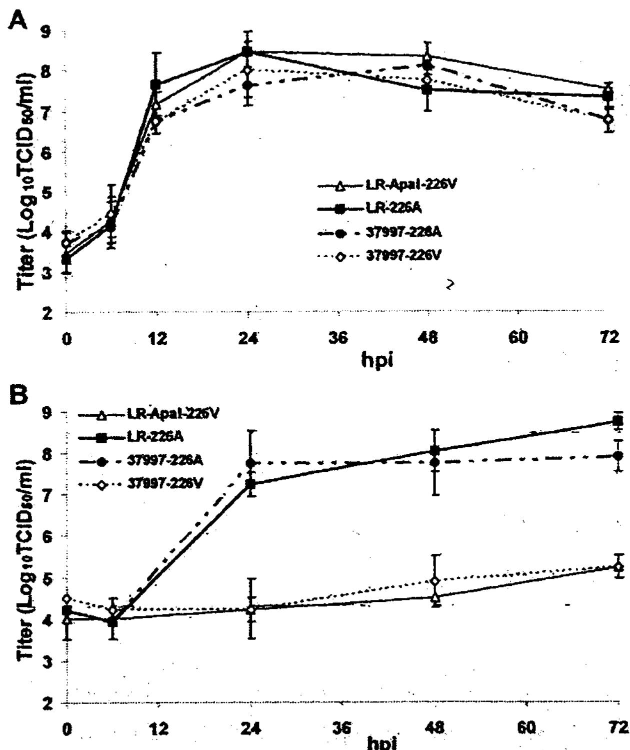

Figure 6. Effect of E1-A226V Mutation on *In Vitro* Growth of CHIKV in Standard (A) and Cholesterol-Depleted (B) C6/36 Cells

Cholesterol-depleted C6/36 cells were produced by five passages in L-15 medium containing 10% FBS treated with 2% CAB-O-Sil for 12 h at room temperature as previously described [52]. Confluent monolayers of standard (A) and cholesterol-depleted (B) C6/36 cells were infected with LR-Apal-226V, LR-226A, 37997-226A and 37997-226V viruses at an MOI of 1.0 (A) and an MOI of 0.1 (B). Cells were washed three times with L-15 medium, and 5.5 ml of fresh L-15 supplied with 10% of standard or CAB-O-Sil-treated FBS were added to the flask. Cells were maintained at 28 °C. At the indicated times post-infection, 0.5 ml of medium was removed and stored at −80 °C for later titration on Vero cells. Viral titers are estimated as average $Log_{10}TCID_{50}$/ml ± standard deviation of two independent experiments.
hpi - hours post-infection.
doi:10.1371/journal.ppat.0030201.g006

with E1-226A viruses. This increased opportunity for *Ae. albopictus* infection, would perpetuate the selection and transmission of the mutant virus.

During transmission competition assays, only E1-226V virus was transmitted to suckling mice by *Ae. albopictus*, although in these experiments, titers of E1-226V and E1-226A viruses were of a high enough magnitude to allow both of these viruses to efficiently infect this mosquitoes species. This indicates that there are additional mechanisms that could ensure evolutional success of the E1-A226V viruses transmitted by *Ae. albopictus*. It is possible that one of these mechanisms is associated with more efficient dissemination of the E1-226V as compared with E1-226A viruses. This could shorten the extrinsic incubation period (EIP)—the time from mosquito infection to transmission—and could have contributed to the evolutionary success of CHIKV during the Reunion outbreak because vectors infected with the LR-226V virus would transmit it more quickly than those infected with LR-226A viruses. Additionally, with relatively short-lived vectors such as mosquitoes [43], longer EIPs reduce transmission efficiency simply because fewer mosquitoes survive long enough to transmit the virus.

Our current studies do not provide data to determine if dissemination efficiency of the E1-226V viruses into the salivary glands is a consequence of more efficient midgut infectivity or if these two phenomena are independent. In this regard, it will be of particular interest to investigate the effect of the E1-A226V mutation on CHIKV transmission by orally or intrathoracically infected *Ae. albopictus* mosquitoes.

Although the CHIKV E1-A226V mutation gives a selective advantage in *Ae. albopictus*, there was not a corresponding advantage in *Ae. aegypti*. The OID_{50} and midgut competition assay data indicate that E1-226V viruses were slightly less infectious for midgut cells of *Ae. aegypti* mosquitoes (Figures 1C, 1D, and 2C; Table 2). Additionally, in contrast to *Ae. albopictus*, E1-226V viruses do not have a detectable advantage for dissemination into salivary glands and heads of *Ae. aegypti*. In transmission competition experiments from *Ae aegypti* to suckling mice, E1-226V conferred a slight competitive advantage over E1-226A (Figure 5C). However, five out of six mice exposed to CHIKV infected *Ae aegypti* had equivalent amounts of both E1-226A and E1-226V viral RNAs. These results are markedly different compared to the results obtained in similar experiments using *Ae. albopictus* mosquitoes and further support the hypothesis that this E1-A226V was specifically selected as a result of adaptation of CHIKV to *Ae. albopictus* mosquitoes. To explain the small fitness advantage associated with the E1-A226V mutation which was observed in transmission experiments, we hypothesize that, similarly to *Ae. albopictus*, E1-226A and E1-226V viruses colonize different *Ae. aegypti* organs at different efficiencies. E1-226A appears to colonize midgut cells of *Ae aegypti* better than E1-226V viruses; however, following dissemination into salivary glands, the E1-226V virus gains an advantage for transmission to vertebrates.

The E1-A226V mutation was found to have a slightly negative effect on infectivity, a negligible effect on dissemination, but a slight positive effect on transmissibility of CHIKV by *Ae. aegypti* in the competition experiment. We suggest that these small (as compared with *Ae. albopictus*) differences associated with the E1-A226V mutation would not be sufficient to have a significant effect on the evolution of CHIKV transmitted by *Ae. aegypti* and would not result in accumulation of this mutation in the regions where *Ae. aegypti* serves as a primary vector for CHIKV. This may explain the lack of emergence of the E1-226V genotype in previous outbreaks and the predominance of E1-226A viruses during the 2006 CHIKV epidemic in India, in which *Ae. aegypti* is considered to be the main vector species [44]. Adaptation of African strains of CHIKV from forest dwelling mosquitoes species to *Ae. aegypti* has never been shown to be associated with any particular mutations, therefore we believe that the same negative impact of E1-A226V would be seen in African mosquito vectors which were responsible for transmission of CHIKV strains ancestral to Reunion isolates.

Our data does not exclude the possibility that the E1-A226V mutation might have a negative effect on the evolution of CHIKV transmitted by *Ae. aegypti*. Since our dissemination and transmission studies were performed using blood meal titers that were 1–2 $Log_{10}TCID_{50}$/ml higher than $Log_{10}OID_{50}$/ml values we suggest that the negative effect of decreased midgut infectivity of E1-A226V on virus trans-

missibility would be almost completely missed, simply because, under this condition, almost 100% of mosquitoes could become infected. In general, CHIKV requires significantly higher blood meal titers for infection of *Ae. aegypti* compared to *Ae. albopictus* [36,37] (Tables 1 and 2), which suggests that the slight decrease in midgut infectivity of E1-226V viruses would have a more profound effect on the evolution of CHIKV transmitted by *Ae. aegypti*, compared to the effect of a small advantage in the ability to compete with E1-226A viruses for transmission to suckling mice. Therefore, if the E1-A226V mutation occurred in CHIKV transmitted by *Ae. aegypti*, it would have a weak negative effect on viral fitness and would most likely not be preferentially selected. Additional experiments are required to evaluate this hypothesis.

Available data cannot exclude the possibility that E1-226A viruses may have an unknown beneficial effect on the fitness of CHIKV in vertebrate hosts over E1-226V viruses, and that the minor negative effect of E1-226A observed in transmission experiments by *Ae. aegypti* can be compensated for by more efficient viral replication in the vertebrate host, leading to an overall more efficient adaptation to the transmission cycle. However, comparison of the different effects of A or V residues at position E1-226 on CHIKV infectivity for, and transmission by *Ae. aegypti* and *Ae. albopictus* mosquitoes clearly suggests that polymorphisms at this position may determine the host range of the alphaviruses and may play an important role in adaptation of the viruses to a particular mosquito vector.

An interesting observation, which should be studied in more detail, was that adaptation of CHIKV to *Ae. albopictus* mosquitoes coincided with the acquisition of CHIKV dependence on cholesterol in the target membrane. It has been previously shown that various mutations in the same region of the E1 protein of SFV and Sindbis virus can modulate the cholesterol dependence of these viruses [33,45] and that SFV independence from cholesterol coincides with more rapid growth of the virus in *Ae. albopictus* [34]. Although there is an apparent association, it is currently unknown if cholesterol dependence of alphaviruses is directly responsible for modulation of fitness of alphaviruses in mosquito vectors. A possible explanation for the opposite effects of the cholesterol-dependent phenotype of SFV and CHIKV on fitness in *Ae. albopictus* may reflect the use of different techniques for mosquito infection. In our study, mosquitoes were orally infected via cholesterol rich blood meals, whereas in the previous study SFV was intrathoracically inoculated into the mosquito [34]. It is also possible that cholesterol-dependent and -independent viruses would replicate differently in different mosquito organs. As such, our data indicate that more efficient colonization of *Ae. albopictus* midgut cells by cholesterol-dependent LR-ApaI-226V is followed by relatively more rapid growth of cholesterol-independent LR -226A virus in mosquito bodies between 3 and 5 dpi (Figure 4B). Three to 5 dpi coincides with virus escape from the mosquito midgut.

Alignment of amino acid sequences that constitute the ij loop of E1 protein from different members of the alphaviruses genus revealed that position E1-226 is not conserved ([33] and data not shown) and can vary even between different strains of the same virus. In this regard, it would be reasonable to determine the cholesterol requirement of other clinically important alphaviruses, especially Venezuelan equine encephalitis virus (VEEV) and eastern equine encephalitis virus (EEEV), which show significant intra-strain variation at position E1-226 among natural isolates of these viruses, and determine mutations which can modulate their cholesterol dependence. In recent studies by Kolokoltsov et al. [46], it was suggested that VEEV, a New world alphavirus, might be cholesterol independent, although the use of Vero cells instead of C6/36 cells, and the use of different protocols for cell membrane cholesterol depletion, make it difficult to compare the results of this study with our findings. Also it would be of interest to determine possible relationships between mutations which modulate cholesterol dependence of alphaviruses other than CHIKV and on their infectivity for *Ae. aegypti* and *Ae. albopictus* mosquitoes and perhaps other epidemiologically important mosquito vectors.

The molecular mechanisms responsible for the association between host range and cholesterol dependence of CHIKV are unknown [47]. It has been proposed that upon exposure to low pH, the E1 protein of cholesterol-dependent viruses senses the target membrane lipid composition and goes through a cholesterol-dependent priming recognition reaction [48] which is not required for cholesterol-independent viruses. It is possible that CHIKV infects *Ae. aegypti* and *Ae. albopictus* midgut cells using different endocytic pathways, which targets virus to cellular compartments with different lipid contents in which fusion occurs. Specific lipids such as cholesterol may differentially affect fusion of cholesterol-dependent and cholesterol-independent CHIKV strains in these compartments and therefore define the outcome of infection. Although our observations are suggestive, more comprehensive studies should be completed to determine the exact molecular mechanisms responsible for penetration of E1-226A and E1-226V viruses into *Ae. aegypti* and *Ae. albopictus* cells.

Although previous laboratory studies have demonstrated susceptibility of *Ae. albopictus* to CHIKV infection [36,37], our data demonstrate that the E1-A226V mutation promoted infection and accelerated dissemination of CHIKV in *Ae. albopictus* mosquitoes and conferred a selective advantage over infection of *Ae. aegypti*. Whilst the mutation did not increase the maximum viral titer attainable in the mosquitoes, the synergistic effects of increased infectivity and faster dissemination of the E1-A226V virus in *Ae. albopictus* would accelerate virus transmission to a naïve human population which would have contributed to initiating and sustaining the 2005–2006 CHIKV epidemic on Reunion island. That a single amino acid change can act through multiple phenotypic effects to create an epidemic situation has implications for other arthropod-transmitted viruses and the evolution of human infectious diseases [49].

Methods

Viruses and plasmids. The viruses and plasmids encoding full-length infectious clones of the LR2006 OPY1 strain CHIK-LR ic (GenBank accession number EU224268; http://www.ncbi.nlm.nih.gov/Genbank/index.html) and GFP-expressing full-length clone LR-GFP-226V (CHIK-LR 5'GFP, GenBank accession number EU224269) have been previously described [15,35]. The plasmids 37997-226A (pCHIK-37997ic, GenBank accession number EU224270) encoding full-length infectious clones of the West African strain of CHIKV 37997 and a GFP-expressing full-length clone 37997-GFP-226A (pCHIK-37997-5GFP, GenBank accession number EU224271) were derived from previously described plasmids pCHIKic and 5'CHIK EGFP [35] by

introducing CHIKV encoding cDNA into a modified pSinRep5 (Invitrogen) at positions 8055–9930. Viruses derived from 37997-226A and 37997-GFP-226A are identical to viruses derived form pCHIKic and 5'CHIK EGFP. To facilitate rapid screening of viruses in mosquitoes, the gene encoding enhance green fluorescent protein (eGFP), that is known not to compromise CHIKV phenotype in mosquitoes [15], was incorporated into clones as previously described [15]. Plasmids were constructed and propagated using conventional PCR-based cloning methods [50]. The entire PCR-generated regions of all constructs were verified by sequence analysis. The maps, sequences and detailed description of the clones are available from the authors upon request. For studies comparing the relative fitness of the mutant (E1-226V) virus and the pre-epidemic genotype (E1-226A), a silent mutation (6454C) was introduced into the CHIK-LR ic, to add an *Apa*I restriction site into the coding sequence of CHIK-LR ic. The resultant plasmid was designated LR-ApaI-226V. The E1-V226A mutation was introduced into CHIK-LR ic and LR-GFP-226V to generate plasmids designated as LR-226A and LR-GFP-226A, respectively. The mutation E1-A226V was also introduced into plasmids 37997-226A and 37997-GFP-226A. The resulted plasmids were designated 37997-226V and 37997-GFP-226V.

All plasmids were purified by centrifugation in CsCl gradients, linearized with *Not*I and *in vitro* transcribed from the minimal SP6 promoter using the mMESSAGE mMACHINE kit (Ambion) following the manufacturer's instructions. The yield and integrity of synthesized RNA were analyzed by agarose gel electrophoresis in the presence of 0.25 μg/ml of ethidium bromide. RNA (10 μg) was transfected into $1x10^7$ BHK-21 cells by electroporation as previously described [15]. Cells were transferred to 25 cm^2 tissue culture flasks with 10 ml of Leibovitz L-15 (L-15) medium, and supernatants were collected at 24 and 48 h post-electroporation and stored at −80 °C. In parallel, $1x10^5$ electroporated BHK-21 cells were serially 10-fold diluted and seeded in six-well plates for infectious centers assay as previously described [15].

Cells and mosquitoes. BHK-21 (baby hamster kidney) cells were maintained at 37 °C in L-15 medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin, and 100 μg/ml streptomycin. C6/36 cells (*Ae. albopictus*) were grown in the same medium at 28 °C. *Ae. aegypti* (white-eyed Higgs variant of the Rexville D strain) and *Ae. albopictus* (Galveston strain) were reared at 27 °C and 80% relative humidity under a 16h light: 8h dark photoperiod, as previously described [35]. Adults were kept in paper cartons supplied with 10% sucrose on cotton balls. To promote egg production females were fed on anaesthetized hamsters once per week.

Rexville D strain of *Ae. aegypti* mosquitoes were originally selected for susceptibility to flavivirus infection [51]. Since there are no known consequences of this original selection with respect to susceptibility to CHIKV, a white eyed variant of the strain that facilitates detection of GFP was used in our experiments.

***In vitro* virus growth of CHIKV in standard and cholesterol-depleted C6/36 cells.** To investigate if the mutation influenced cholesterol dependence of the virus, cholesterol-depleted C6/36 cells were prepared by five passages in L-15 medium containing 10% FBS treated with 2% CAB-O-Sil (Acros Organics) for 12 h at room temperature as previously described [52]. CHIKV growth curves were determined by infecting cholesterol-depleted and normal C6/36 cells at a multiplicity of infection (MOI) of 0.1 and 1.0, respectively, by rocking for 1 h at 25 °C. The cells were washed three times with L-15 medium and 5.5 ml of fresh L-15 supplied with 10% of standard or CAB-O-Sil treated FBS was added to the flask. At the indicated times post-infection, 0.5 ml of medium was removed and stored at −80 °C until titrated. The volume of medium was then restored by adding 0.5 ml of appropriate medium.

Titrations. Viral titers from mosquito samples and from tissue culture supernatant were determined using Vero cells and expressed as tissue culture infectious dose 50 percent endpoint titers ($Log_{10}TCID_{50}$) as previously described [53]. Additionally, for viral competition experiments, titers of LR-Apa-226V LR-226A viruses were determined using standard plaque assay on Vero cells as previously described [54].

Oral infection of mosquitoes. *Ae. aegypti* and *Ae. albopictus* were infected in an Arthropod Containment Level 3 insectary as described previously [35,55]. To make infectious blood meals for the viruses lacking eGFP, viral stocks derived from electroporated BHK-21 cells were mixed with an equal volume of defibrinated sheep blood and supplemented with 3 mM ATP as a phago-stimulant. To produce infectious blood meals for the eGFP-expressing viruses, the viruses were additionally passed on BHK-21 cells. The cells were infected at a MOI ~ 1.0 with virus derived from electroporation. At 2 dpi, cell culture supernatants were mixed with an equal volume of defibrinated sheep blood and presented to 4- to 5-day-old female mosquitoes that had been starved for 24 h, using a Hemotek membrane feeding system (Discovery Workshops) and hamster skin membrane. Mosquitoes were allowed to feed for 45 min, and engorged mosquitoes (stage ≥3+ [56]) were sorted and returned to a cage for maintenance. Blood meals and three to four mosquitoes were immediately removed for titration and/or RNA extraction. Depending on the purpose of the experiments, mosquitoes were collected at different days post-infection and either titrated to determine viral titer, dissected for analysis of eGFP expression in the midguts or salivary glands [15], or used for RNA extraction in competition experiments.

To estimate the Oral Infectious Dose 50% values (OID_{50}), serial 10-fold dilutions of viruses were made in L-15 medium followed by mixing the samples with defibrinated sheep blood. Mosquitoes were dissected at 7 dpi and eGFP expression in infected midguts was analyzed by fluorescence microscopy. A mosquito was considered infected if at least one foci of eGFP-expressing cells was present in the midgut. The experiments were performed twice for each virus. OID_{50} values and confidence intervals were calculated using PriProbit (version 1.63).

Viral competition experiments. To test the hypothesis that the E1-A226V mutation might be associated with a competitive advantage in mosquito vectors, competition assays were designed similar to those described previously in mice [57], with minor modifications (Figure 2A). Both *Ae. aegypti* and *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 plaque-forming units (pfu)/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. It had been previously found that for these two viruses the ratio of viral RNAs corresponds to the ratio of viral titers (data not shown). Midguts were collected at 7 dpi and analyzed in pools of eight to ten, and heads were collected at 12 dpi and analyzed in pools of five. RNA was extracted from the tissue pools using TRIzol reagent (Invitrogen) followed by additional purification using a Viral RNA mini kit (QIAGEN). RNAs from blood meal samples were extracted using Viral RNA Mini Kit followed by treatment with DNAse (Ambion) to destroy any residual plasmid DNA contaminant in the viral samples. RNA was reversed transcribed from random hexamer primers using Superscript III (Invitrogen) according to the manufacturer's instructions. cDNA was amplified from 41855ns-F5 (5'- ATATCTAGACATGGTGGAC) and 41855ns-R1 (5'-TATCAAAGGAGGCTATGTC) primers using Taq DNA polymerase (New England Biolabs). PCR products were purified using Zymo clean columns (Zymo Research) and were quantified by spectrophotometry. Equal amount of PCR products were digested with *Apa*I, separated in 2% agarose gels that were stained using ethidium bromide. Thus the LR-Apa-226V and LR-226A viruses could be distinguished by size on an agarose gel (Figure 2A). Gel images were analyzed using TolaLab (version 2.01). Relative fitness of LR-Apa-226V and LR-226A viruses was calculated as a ratio between 226V and 226A bands in the sample, divided by the control ratio of 226V and 226A in the blood meal.

Virus competition in an animal transmission model. *Ae. aegypti* and *Ae. albopictus* mosquitoes were presented with a blood meal containing 10^7 pfu/ml of LR-Apa-226V and 10^7 pfu/ml of LR-226A viruses. At 13 dpi, ten to 15 mosquitoes were placed in separate paper cartons and starved for 24 h. The next day the mosquitoes in each carton were presented with individual 2- to 3-day-old suckling mouse (Swiss Webster). Feeding continued until 2–3 mosquitoes per carton were fully engorged (stage ≥3+[56]). In a parallel experiment six 2- to 3-day-old suckling mice were subcutaneously infected with 20 μl of mixture containing ~ 25 pfu of LR-Apa-226V and ~ 25 pfu of LR-226A viruses. Mice were returned to their cage and sacrificed on day 3 post-exposure. Blood from each individual mouse (~ 50 μl) was collected and immediately mixed with 450 μl of TRIzol reagent for RNA extraction. The RNA was processed as described above. All animal manipulations were conducted in accordance with federal laws, regulations, and in compliance with National Institutes of Health and University of Texas Medical Branch Institutional Animal Care and Use Committee guidelines and with the Association for Assessment and Accreditation of Laboratory Animal Care standards.

Supporting Information

Figure S1. Schematic Representation of the Viruses Used in This Study

Found at doi:10.1371/journal.ppat.0030201.sg001 (917 KB PDF).

Figure S2. Growth of the eGFP-Expressing Viruses in BHK-21(A, C) and C6/36 (B, D) Cells

Confluent monolayers of BHK-21 and C6/36 cells in T25 tissue culture flacks were infected with LR-GFP-226V and LR-GFP-226A (A,

B) or 37997-GFP-226A and 37997-GFP-226V viruses derived from electroporation at a MOI of 0.1. At the indicated times post-infection, 0.5 ml of medium was removed and stored at −80 °C for later titration on Vero cells. Viral titers are expressed as $Log_{10}TCID_{50}$/mL

Found at doi:10.1371/journal.ppat.0030201.sg002 (372 KB PDF).

Figure S3. Growth of the CHIK-LR ic, LR-ApaI-226V and LR-226A Viruses in BHK-21(A) and C6/36 (B) Cells

Confluent monolayers of BHK-21 and C6/36 cells in T25 tissue culture flacks were infected with LR-GFP-226V and LR-GFP-226A (A, B) or 37997-GFP-226A and 37997-GFP-226V viruses derived from electroporation at a MOI of 1.0. At the indicated times post-infection, 0.5 ml of medium was removed and stored at −80 °C until titrated on Vero cells. Viral titers are expressed as $Log_{10}TCID_{50}$/ml ± standard deviation of three independent experiments.
hpi - hours post-infection.

Found at doi:10.1371/journal.ppat.0030201.sg003 (177 KB PDF).

Figure S4. Competition between CHIK-LR ic and LR-ApaI-226V for Growth in BHK-21 and C6/36 Cells

Cells were infected with a 1:1 mixture of both viruses at a MOI of 0.001. 2 dpi, cell culture supernatant was collected and samples proceeded as described. The experiment was repeated three times for each of the cell types.
inoc - initial ratio of CHIK-LR ic and LR-ApaI-226V in the inoculum used for infection of cells.
Relative fitness (RF) of CHIK-LR ic and LR-ApaI-226V was calculated as an average ratio between CHIK-LR ic and LR-ApaI-226V bands in the supernatant obtained from BHK-21 cells (RF_1) and C6/36 cells (RF_2), divided by the control ratio between CHIK-LR ic and LR-ApaI-226V in the inoculum.

Found at doi:10.1371/journal.ppat.0030201.sg004 (3.6 MB PDF).

Table S1. Specific Infectivity and Virus Titers after Electroporation

a - amino acids at position of E1-226.
b - Specific infectivity of *in vitro* transcribed RNA. 10^7 BHK-21 cells were transfected with 10 µg of RNA. Electroporated BHK-21 cells were 10-fold serially diluted, seeded in 6-well tissue culture plates containing $5x10^5$ naive BHK-21 cells per well and covered with 0.5% agarose in L-15. Plaques were scored on day 2 post-transfection.
c - Supernatants of electroporated BHK-21 cells were collected on days 1 and 2. Virus titers were determined by titration on Vero cells and expressed as $Log_{10}TCID_{50}$/ml.
hpi - hours post-infection.

Found at doi:10.1371/journal.ppat.0030201.st001 (34 KB DOC).

Table S2. Infection Rates and Average Titers of CHIKV-LR ic or LR-ApaI-226V in Orally Infected *Ae. aegypti* and *Ae. albopictus*

Ae. aegypti mosquitoes were orally presented with 7.24±0.4 $Log_{10}TCID_{50}$/ml of CHIKV-LR ic (summary of two experiments) and 6.52 $Log_{10}TCID_{50}$/ml of LR-ApaI-226V.
Ae. albopictus mosquitoes were orally presented with 7.24±0.4 $Log_{10}TCID_{50}$/ml of CHIKV-LR ic (summary of two experiments) and 7.52 $Log_{10}TCID_{50}$/ml LR-ApaI-226V.
At 7 and 14 dpi, mosquitoes were collected and triturated in 1mL of L-15 medium for titration on Vero cells.
Titers are reported as $Log_{10}TCID_{50}$/ml ± standard deviation.

Found at doi:10.1371/journal.ppat.0030201.st002 (31 KB DOC).

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Author contributions. KAT and SH conceived and designed the experiments. KAT, DLV, and CEM performed the experiments and analyzed the data. KAT, DLV, CEM, and SH wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.

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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 2007. 10. 5	新医薬品等の区分 該当なし	機構処理欄
一般的名称	人赤血球濃厚液		研究報告の公表状況	ProMED 20071001-3237, 2007 Oct 1. 情報源:[1]China Daily, Xinhua News Agency report, 2007 Sep 30. [2]VietNamNet Bridge, 2007 Sep 26 [3]Daily Times, 2007 Sep 27. [4]Associated Press, 2007 Sep 29.	公表国 [1]中国[2]ベトナム[3]パキスタン[4]汎米保健機構	
販売名(企業名)	赤血球濃厚液-LR「日赤」(日本赤十字社) 照射赤血球濃厚液-LR「日赤」(日本赤十字社)					

研究報告の概要

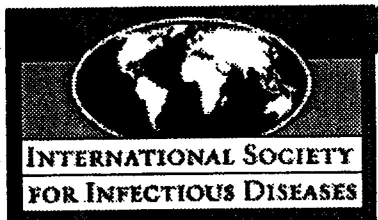
○デングウイルス最新情報
[1]中国(福建省):9月30日、保健当局は福建省莆田市でデング熱症例39例を確認したと発表した。感染拡大を防ぐ為の総合的予防対策が実施され、医療機関でのモニタリングが強化されている。市民には、蚊の増殖を防ぐ為に衛生状態改善が呼びかけられている。
[2]ベトナム:2007年のデング熱発生件数は、昨年と比べて50%増加したと保健省が報告した。9月24日時点で患者68,000人が報告され、60人が死亡した。感染例のほとんどは南部で発生している。患者は通常10歳以下の子どもが多いが、2007年は成人患者も増加している。
[3]パキスタン(カラチ):保健省のデング熱サーベイランス班によると、カラチ市の4つの病院で22例の新規デング熱疑い症例が報告された。うち20人が陽性、2人が検査中となっている。2007年はこれまでに170例の疑い症例が報告された。
[4]ラテンアメリカ:デング熱がラテンアメリカとカリブ海諸国に感染拡大しており、この10年で最も深刻な事態になっている。2007年はこれまでに630,356人の患者が主にブラジル・ベネズエラ・コロンビアから報告され、うち12,147人が出血熱を発症、183人が死亡した。このまま拡大が続けば2002年の1,015,000例を超える可能性がある。流行が沈静化しないと社会的、経済的に大きな影響が出るだろうと汎米保健機構の専門家は述べている。観光や移住によって4系統のウイルス株が地域内で循環しているために、患者が重症化しやすくなっていると考えられている。

使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-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
中国福建省、ベトナム、パキスタン、ラテンアメリカでデング熱が流行しているとの報告である。	日本赤十字社では、輸血感染症対策として問診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。問診でデング熱の既往があった場合には、治癒後1ヶ月間献血不可としている。今後も引き続き情報の収集に努める。



Archive Number 20071001.3237
Published Date 01-OCT-2007
Subject PRO/EDR> Dengue/DHF update 2007 (37)

DENGUE/DHF UPDATE 2007 (37)
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A ProMED-mail post
<http://www.promedmail.org>
ProMED-mail is a program of the
International Society for Infectious Diseases
<http://www.isid.org>

In this update:
[1] China (Fujian)
[2] Viet Nam
[3] Pakistan (Karachi)
[4] Latin America

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[1] China (Fujian)
Date: Sun 30 Sep 2007
Source: China Daily, Xinhua News Agency report [edited]
<http://www.chinadaily.com.cn/china/2007-09/30/content_6149071.htm>

On Sunday [30 Sep 2007], health authorities said 39 dengue fever cases have been confirmed in Putian City of east China's Fujian Province. Thus far, 26 of the 39 patients in Hanjiang District of Putian City have been cured and the others are in stable condition, said the provincial health department.

The city has adopted "comprehensive prevention and control measures" to curb the spread of the disease, said the department. All medical and health institutions in the province have also strengthened monitoring on the disease, it added.

The department reminded citizens of household sanitation and the prevention of proliferation of mosquitoes, which transmit the disease [\ us]

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Communicated by:
ProMED-mail Rapporteur Brent Barrett

[Putian City is situated in the central part of the coastal area of Fujian Province. Putian neighbors Fuzhou in the northeast and Quanzhou in the southeast, and is separated from Taiwan by the Taiwan Strait.

[A zoomable map of Fujian Province showing the location of Putian city can be accessed at
<http://encarta.msn.com/map_701510630/Fujian.html>. - Mod.TY]

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[2] Viet Nam
Date: Wed 26 Sep 2007
Source: VietNamNet Bridge [edited]
<http://english.vietnamnet.vn/social/2007/09/745035/>

The incidence of dengue fever in Viet Nam has risen by almost 50 percent this year [2007] against last year [2006], reports the Health Ministı

A medical worker instructs Dao ethnic minorities in the northern mountainous province of Yen Bai's Quang Minh Commune to dip mosquito

nets in chemicals to prevent dengue fever.

About 68 000 people had been stricken with the mosquito-borne disease, Preventative Health Department director Nguyen Huy Nga said on Monday [24 Sep 2007]; 60 had died.

Most infections had occurred in southern Dong Thap, An Giang, Tien Giang, and Ben Tre provinces and the total increase was about 48 percent, he said.

Ho Chi Minh [HCM] City-based Pasteur Institute National Dengue Fever Programme representative Luong Chan Quang said more than 58 000 people had been infected in the Cuu Long (Mekong) Delta provinces by the end of August [2007]. Deaths were put at 54-40 percent more than last year [2006].

Infections in Tien Giang Province totalled 9800 with 9 deaths, Dong Thap 8700 with 9 deaths, and An Giang 6000 with 6 deaths.

In HCM City, almost 5400 people had been stricken with dengue fever -- 40 percent more than last year [2006 -- and 6 had died.

Quang warned that another serious outbreak was likely in the southern delta before the end of the year [2007] if effective preventive measures were not taken because people regularly stored water to prepare for the dry season.

The _Aedes_ mosquito, which carries dengue fever, breeds in still or stagnant water.

HCM City Preventive Health Department deputy director Nguyen Dac Tho said about 350 people were being admitted to hospital each week with dengue fever -- 50 more than last year [2006].

Inner city districts 8, 10, 11, Binh Thanh, and Binh Tan were the worst affected. People in densely populated precincts stored more water for their own use than others as did construction projects, said Dr Tho.

Dengue fever is most common among children under 10 but the number of afflicted adults has increased this year [2007].

HCM City Tropical Diseases Hospital figures show that of about 150 people admitted to the hospital with dengue fever each week, more than 100 were adults.

There are 4 types of the dengue fever virus that often result in similar symptoms. This year [2007], the transmitted virus was usually type 1 or type 2.

Haemorrhagic fever is a severe, often fatal, complication of dengue fever.

The HCM City People's Committee has mobilised measures to prevent dengue fever across the city. Citizens are encouraged to clean around their residences every Sunday and spray mosquito killer [insecticides].

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[A map of Viet Nam can be accessed at
<http://www.lib.utexas.edu/maps/middle_east_and_asia/vietnam_admin01.jpg>.
- Mod.TY]

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[3] Pakistan (Karachi)
Date: Thu 27 Sep 2007
Source: Daily Times [edited]
<http://www.dailytimes.com.pk/default.asp?page=2007%5C09%5C27%5Cstory_27-9-200

The Sindh Health Department's Dengue Fever Surveillance Cell reported 22 fresh cases of the disease in select hospitals across Karachi on Wednesday [26 Sep 2007].

Additional health secretary and in-charge of the surveillance cell, Dr Shakil Malik, giving details of these cases, told APP [Associated Press of Pakistan] that 20 of the patients are positive and they are waiting for the report on the other 2.

The hospitals that dispatched reports include Liaquat National Hospital, Ziauddin Hospital, Bismillah Taqee Hospital, and Zainab Panjwani Hospital. "Since we just reactivated the cell on Tuesday [25 Sep 2007], it will take time before we make contact with all the hospitals scattered across the city," he said. To a question, he said that around 170 suspected cases of dengue fever have been reported from across the city this year -- from January [2007] to date. He also referred to the report the provincial health department received from a local laboratory (Mid Citi Lab) that tested 24 OPD [out patient department] patients between August [2007] and now. Of these individuals, 12 came out positive.

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Communicated by:
ProMED Rapporteur Brent Barrett

[Karachi is located on the Arabian Sea. A map of Pakistan can be accessed at
<http://www.lib.utexas.edu/maps/middle_east_and_asia/pakistan_pol_2002.jpg>.
- Mod.TY]

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[4] Latin America
Date: Sat 29 Sep 2007
Source: Associated Press [edited]
<http://ap.google.com/article/ALeqM5i86GcnUASvmXnPi9bBXcqngijdSQD8RVA16O4>

Dengue fever is spreading across Latin America and the Caribbean in one of the worst outbreaks in decades, causing agonizing joint pain for hundreds of thousands of people and killing nearly 200 so far this year [2007].

The mosquitoes that carry dengue are thriving in expanded urban slums scattered with water-collecting trash and old tires. Experts say dengue is approaching record levels this year [2007] as many countries enter their wettest months.

"If we do not slow it down, it will intensify and take a greater social and economic toll on these countries," said Dr. Jose Luis San Martin, head of anti-dengue efforts for the Pan American Health Organization (PAHO), a regional public health agency.

The US Centers for Disease Control and Prevention (CDC) in Atlanta has posted advisories this year [2007] for people visiting Latin American and Caribbean destinations to use mosquito repellant and stay inside screened areas whenever possible.

"The danger is that the doctors at home don't recognize the dengue," said Dr. Wellington Sun, the chief of the CDC's dengue branch in San Juan, [Puerto Rico] "The doctors need to raise their level of suspicion for any traveler who returns with a fever."

Dengue has already damaged the economies of countries across the region by driving away tourists, according to a document prepared for a PAHO conference beginning Monday [1 Oct 2007] in Washington.

Some countries have focused mosquito eradication efforts on areas popular with tourists. Mexico sent hundreds of workers to the resorts of Puerto Vallarta, Cancun, and Acapulco this year [2007] to try to avert outbreaks.

Health ministers from across the region meet at the PAHO conference and San Martin said he will urge them to devote more resources to dengue feve

The tropical virus was once thought to have been nearly eliminated from Latin America, but it has steadily gained strength since the early 1980s. Now, officials fear it could emerge as a pandemic similar to one that became a leading killer of children in Southeast Asia following World War II.

Officials say the virus is likely to grow deadlier in part because tourism and migration are circulating 4 different strains across the region. A person exposed to one strain may develop immunity to that strain -- but subsequent exposure to another strain makes it more likely the person will develop the hemorrhagic form.

"The main concern is what's happening in the Americas will recapitulate what has happened in Southeast Asia, and we will start seeing more and more severe types of cases of dengue as time progresses," Sun said.

So far this year [2007], 630 356 dengue cases have been reported in the Americas -- most in Brazil, Venezuela, or Colombia -- with 12 147 cases of hemorrhagic fever and 183 deaths, according to the Pan American Health Organization. With the spread expected to accelerate during the upcoming rainy season in many countries, cases this year [2007] could exceed the 1 015 000 reported in 2002, according to San Martin.

In Puerto Rico, where 5592 suspected cases and 3 deaths have been reported, some lawmakers called this week for the health secretary to resign.

In the Dominican Republic, which has reported 25 deaths this year [2007], the health department announced Thursday [27 Sep 2007] that it would train 2.5 million public school students to encourage parents and neighbors to eliminate standing water.

Researchers have not yet developed a vaccine against dengue and Sun said that for now, the only way to stop the virus is to contain the mosquito population -- a task that relies of countless, relentless individual efforts including installing screen doors and making sure mosquitoes are not breeding in garbage.

"It's like telling people to stop smoking," he said. "They may do it for a while, but they don't do it on a consistent basis and without doing that, it's not effective."

While dengue is increasing around the developing world, the problem is most dramatic in the Americas, according to the CDC.

Health officials believe the resurgence of the malaria-like illness is due partly to a premature easing of eradication programs in the 1970s.

Migration and tourism also have carried new strains of the virus across national borders, even into the United States, which had largely wiped out the disease after a 1922 outbreak that infected a half-million people.

Mexico has been struggling with an alarming increase in the deadly hemorrhagic form of dengue, which now accounts for roughly one in 4 cases. The government has confirmed 3249 cases of hemorrhagic dengue for the year through 15 Sep [2007], up from 1924 last year [2006].

The CDC says there is no drug to treat hemorrhagic dengue, but proper treatment, including rest, fluids, and pain relief, can reduce death rates to about one percent.

San Martin said he use the meetings starting Monday [1 Oct 2007] to urge enforcement of trash disposal regulations, more investment in mosquito control and new incentives for communities to participate. "It is a battle of every government, every community and every individual," he said.

[Byline: Michael Melia]

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The WHO (World Health Organisation) description of dengue fever and the more deadly dengue hemorrhagic fever [DHF] can be found in ProMED-mail's "Dengue/DHF update 20070514.1541". ProMED-mail thanks the contributors to this update and encourages others to contribute reports also. - Mod.TY] 97

[see also:
Dengue/DHF update 2007 (36) 20070924.3165
Dengue/DHF update 2007 (35) 20070918.3103
Dengue/DHF update 2007 (34) 20070908.2964
Dengue/DHF update 2007 (33) 20070821.2726
Dengue/DHF update 2007 (32) 20070816.2675
Dengue/DHF update 2007 (31) 20070806.2555
Dengue/DHF update 2007 (30) 20070730.2440
Dengue/DHF update 2007 (20) 20070514.1541
Dengue/DHF update 2007 (10) 20070225.0683
Dengue/DHF update 2007 (01) 20070103.0030]
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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 2007. 10. 16	新医薬品等の区分 該当なし	機構処理欄
一般的名称	人赤血球濃厚液		研究報告の公表状況	Yahoo!ニュース. 2007 Oct 14.	公表国 台湾	
販売名(企業名)	赤血球濃厚液-LR「日赤」(日本赤十字社) 照射赤血球濃厚液-LR「日赤」(日本赤十字社)					

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
○台南で511人が感染！猛威をふるうデング熱、「蚊」撲滅作戦に軍も動員へ—台湾南部 台湾のニュースサイト「中国台湾網」などが伝えたところによると、台湾南部でデング熱が流行。台南市政府の最新の調査では、2007年10月13日までに市内で511人の感染者が確認されたほか、隣接する高雄市でも2つの区で集団感染が発生しており、感染の広がりは過去最大規模。 高雄市では来週、スポーツ競技大会が予定されており、選手団の感染を防ぐため競技会場と選手村周辺地域を警戒重点区域に指定した。市職員のほか、軍も動員し、デング熱ウイルスを媒介する蚊の撲滅作戦を展開する方針だ。	赤血球濃厚液-LR「日赤」 照射赤血球濃厚液-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
台湾南部でデング熱が流行し、台南市内で511人の感染者が確認されたほか、隣接する高雄市でも集団感染が発生しており、感染の広がりは過去最大規模となっているとの報告である。	日本赤十字社では、輸血感染症対策として問診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。問診でデング熱の既往があった場合には、治癒後1ヶ月間献血不可としている。今後も引き続き情報の収集に努める。

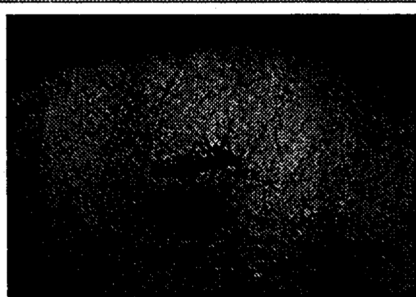
4,999円までヤフオクの入札が全員参加無料！　Yahoo!検索 検索

[PR]　まず、あなたが「一生添い遂げたい人」を診断してみませんか！[無料]

海外　文字サイズ：小 中 大

台南で511人が感染！猛威をふるうデング熱、「蚊」撲滅作戦に軍も動員へ―台湾南部

10月14日9時38分配信 Record China



拡大写真

台湾南部で蚊が媒介するデング熱が大流行。10月13日までに台南市で511人の感染が確認されたほか、高雄市でも集団感染が発生。行政と軍が協力して大規模な蚊の撲滅作戦を展開する方針。

台湾のニュースサイト「中国台湾網」などが伝えたところによると、台湾南部でデング熱が流行。台南市政府の最新の調査では、2007年10月13日までに市内で511人の感染者が確認されたほか、隣接する高雄市でも2つの区で集団感染が発生しており、感染の広がりは過去最大規模。

高雄市では来週、スポーツ競技大会が予定されており、選手団の感染を防ぐため競技会場と選手村周辺地域を警戒重点区域に指定した。市職員のほか、軍も動員し、デング熱ウイルスを媒介する蚊の撲滅作戦を展開する方針だ。（翻訳・編集/本郷智子）

最終更新:10月14日9時38分

Record China

ソーシャルブックマークへ投稿 5件：

（ソーシャルブックマークとは）

Yahoo!知恵袋に質問する

関連トピックス　台湾

フレッシュアイニュース　デング熱 に関する　ブログ　デング熱とは？

みんなの感想　この話題についてみんながどう感じたかわかります。

PR

海外トピックス

英、五輪予定地で火災と黒煙
NY劇場スト 1日19億円の損失
サウジ王子「空飛ぶ宮殿」購入 NEW
ベトナム洪水 ワニ数百匹脱走
ベネズエラ大統領、叱られる
ブット元首相を再び軟禁下に NEW
ヒラリー陣営がやらせ質問

バックナンバー

注目の情報

車の現在価値は？
複数社の査定額を比較できるcarview料査定なら愛車の最高価格がわかる

40歳には見えない
全然同じ歳に見えない彼女に聞いた女性が満足しているコスメのお話≫

nikkeiBPnet on Yahoo!ニュース

ドコモが1円端末と決別、携帯ビジネスモデルが変わる NEW

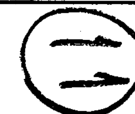
nikkeiBPnet on Yahoo!ニューストップ

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	機構処理欄
				2007. 10. 22	該当なし	
一般的名称	人赤血球濃厚液		研究報告の公表状況	三浦左千夫, 肥後廣夫, 竹内勤. 第48回日本熱帯医学会大会	公表国 日本	
販売名(企業名)	赤血球濃厚液-LR「日赤」(日本赤十字社) 照射赤血球濃厚液-LR「日赤」(日本赤十字社)					

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
○日本におけるラテンアメリカ人の慢性シャーガス病キャリアーからの献血についての対策検討 近年ラテンアメリカからの就労目的の定住化人口が増加の一途にあり、既に40万人を超えようとしている。当然就労目的のため、表向きは健常者としての来日である。しかし、就労中に疲れを訴え呼吸困難などの不調を来たした為に医療機関を受診し、出身地を考慮の後、血清免疫学的検査の結果シャーガス病感染を示唆された者が13名見いだされた。中には、末梢血で病原体*Trypanosoma cruzi*(*T. cruzi*)の存在を示唆する*T. cruzi* DNAのPCR増幅断片が、検査の度に検出される者もいた。また血液培養(LIT培地、NNN培地)で血液型虫体の分離にも成功した例など、慢性シャーガス病即ち病原体のキャリアーであることが明らかとなった者もいた。これらの抗体陽性者13名のうち1名については日本滞在中に献血を行っていた。注意すべきは、我が国では主要媒介昆虫が棲息しないものの、シャーガス病慢性キャリアーからの輸血、臓器移植などによる二次的感染の危険性である。今回、献血機関で保存血用に使用しているカーミC液(CPD液)を用いて感染マウス血液を4℃にて1～21日間保存処理を行った。これを正常マウスに接種し感染性、病原性について基礎的な検討を行った。その結果マウスへの感染性は無処置のものと差異は無かったが、病原性についてはかなり減弱していることが示唆された。これは*T. cruzi*に対して4℃という低温ストレスが影響したものと考えられる。また同時に白血球除去フィルターを用いての*T. cruzi*の通過性など検討した。その結果殆どのフィルターを*T. cruzi*虫体は通過してしまった。従って、残念ながら我が国で行われている現在の輸血用の保存血液提供システムでは、シャーガス病の輸血感染を確実には防止できない。ラテンアメリカ人に対する抗体チェックの実施とその強化などを、社会医学的影響を考慮の上、今後も更に安全輸血業務を遂行する為の対策の改善を図る必要がある。	赤血球濃厚液-LR「日赤」 照射赤血球濃厚液-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
カーミC液(CPD液)を用いて*T. cruzi*感染マウス血液を4℃にて1～21日間保存処理を行ったところ、マウスへの感染性は無処置のものと差異は無かったが、病原性はかなり減弱していることが示唆された。我が国においても、安全輸血業務を遂行する為の対策の改善を図る必要があるとの報告である。	日本赤十字社は、輸血感染症対策として献血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、シャーガス病の既往がある場合には献血不適としている。日本在住の中南米出身献血者については、国と協議しつつ対応を検討中である。今後も引き続き情報の収集に努める。



12C－02

日本におけるラテンアメリカ人の慢性シャーガス病キャリアーからの献血についての対策検討

The study of the counter measuers against blood donation from chronic Chagas disease carrier of latin-american residing in Japan

三浦　左千夫[1]、肥後　廣夫[2]、竹内　勤[1]
慶応義塾大学医学部熱帯医学寄生虫[1]、九州大学医学部感染免疫熱帯医学分野[2]

近年ラテンアメリカからの就労目的の定住化人口が増加の一途にあり、既に40万人を超えようとしている。当然就労目的の為、表向きは健常者としての来日である。しかし、就労中に疲れを訴え呼吸困難などの不調を来たした為に医療機関を受診し、出身地を考慮の後、血清免疫学的検査の結果シャーガス病感染を示唆された者が13名見いだされた。中には、末梢血で病原体*Trypanosoma cruzi*（*T.cruzi*）の存在を示唆する*T.cruzi*-DNAのPCR増幅断片が、検査のたびに検出される者もいた。また血液培養（LIT培地、NNN培地）で血液型虫体の分離にも成功した例など、慢性シャーガス病即ち病原体のキャリアーであることが明らかとなった者もいた。これらの抗体陽性者13名のうち１名については日本滞在中に献血を行っていた。注意すべきは、我が国では主要媒介昆虫が棲息しないものの、シャーガス病慢性キャリアーからの輸血、臓器移植などによる二次的感染の危険性である。今回、献血機関で保存血用に使用しているカーミＣ液（CPD液）を用いて感染マウス血液を４℃にて１～21日間保存処理をおこなった。これを正常マウスに接種し感染性、病原性ついて基礎的な検討を行った。その結果マウスへの感染性は無処置のものと差異は無かったが、病原性についてはかなり減弱していることが示唆された。これは*T.cruzi* に対して４℃という低温ストレスが影響したものと考えられる。　また同時に白血球除去フィルターを用いての*T.cruzi*の通過性など検討した。その結果殆どのフィルターを*T.cruzi*虫体は通過してしまった。従って、残念ながら我が国で行われている現在の輸血用の保存血液提供システムでは、シャーガス病の輸血感染を確実には防止できない。ラテンアメリカ人に対する抗体チェックの実施とその強化などを、社会医学的影響を考慮の上、今後も更に安全輸血業務を遂行する為の対策の改善を図る必要がある。

別紙様式第2-1

No. 19

医薬品 研究報告 調査報告書

<table>
<tr><td>識別番号・報告回数</td><td></td><td></td><td>報告日</td><td>第一報入手日
2007. 10. 22</td><td>新医薬品等の区分
該当なし</td><td rowspan="3">機構処理欄</td></tr>
<tr><td>一般的名称</td><td colspan="2">人赤血球濃厚液</td><td rowspan="2">研究報告の公表状況</td><td rowspan="2">Kleinman SH, Glynn SA, Lee TH, Tobler L, Montalvo L, Todd D, Kiss JE, Shyamala V, Busch MP; National Heart, Lung, Blood Institute Retrovirus Epidemiology Donor Study (REDS-II). Transfusion. 2007 Oct;47(10):1756-64.</td><td rowspan="2">公表国
米国</td></tr>
<tr><td>販売名(企業名)</td><td colspan="2">赤血球濃厚液-LR「日赤」(日本赤十字社)
照射赤血球濃厚液-LR「日赤」(日本赤十字社)</td></tr>
<tr><td>研究報告の概要</td><td colspan="5">○供血者のパルボ・ウイルスB19DNAの高感度PCRスクリーニング法による陽性率と定量測定
背景:供血者における高感度核酸検査法を用いたパルボ・ウイルスB19 DNAの陽性率は、血漿分画製剤製造部門で高力価ウイルスを検出するためにデザインされた検査の検出率よりも高いことが最近示されている。
試験デザインおよび方法:米国の血液センター7施設において2000年～2003年の期間に採取した5020名の供血血液から得られた保存血漿検体を検査した。50%検出限界(LOD)1.6 IU/mL(95%信頼区間 [CI], 1.2～2.1 IU/mL)及び95% LOD 16.5 IU/ mL (95% CI, 10.6～33.9 IU/mL)のリアルタイムB19 DNA PCR法(PCR; TaqMan, Applied Biosystems)を用いて検査を実施した。B19 DNAの確認と測定は、別の2つの検体の再検査により行った。陽性が確定した検体は、FDAが承認した検査法を用いて抗B19免疫グロブリンM(IgM)及びIgGの有無をテストした。
結果:B19 DNA陽性率は0.88%(95% CI, 0.64%～1.2%)であった。B19 DNA力価が20 IU/mL以上であった供血者23名のDNA値は、中央値が105 IU/mL(四分位範囲42～481 IU/mL)であり、最高値が1869 IU/mLであった。B19 DNA陽性供血はいずれもIgG陽性であり、そのうちの10名(23%)はIgMも陽性であった。血清中のIgMが陽性であることは、DNA値の増加と関連付けられた(p = 0.0013)。
結論:供血者のほぼ1%に低値のB19 DNAが検出された。IgM 及びIgG B19抗体のいずれも陽性であったDNA陽性供血(23%)は、急性感染症である可能性が高く、IgGが陽性であるがIgMが陰性の供血は、持続性のB19感染である可能性が高い。</td><td rowspan="3">使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-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</td></tr>
<tr><td colspan="3">報告企業の意見</td><td colspan="3">今後の対応</td></tr>
<tr><td colspan="3">米国の供血者のほぼ1%に低値のヒトパルボウイルスB19 DNAが検出されたとの報告である。</td><td colspan="3">今後も引き続き、ヒトパルボウイルスB19に関する新たな知見及び情報の収集に努める。日本赤十字社では、以前よりRHA法によるB19抗原検査を導入、ウイルス量の多い血液を排除している。今後は検査方法の改善によりさらなる感度向上を目指すこととしている。</td></tr>
</table>

(12)

TRANSFUSION COMPLICATIONS

Prevalence and quantitation of parvovirus B19 DNA levels in blood donors with a sensitive polymerase chain reaction screening assay

Steven H. Kleinman, Simone A. Glynn, Tzong-Hae Lee, Leslie Tobler, Leilani Montalvo, Deborah Todd, Joseph E. Kiss, Venkatakrishna Shyamala, and Michael P. Busch for the National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study (REDS-II)

BACKGROUND: Blood donor parvovirus B19 DNA prevalence with sensitive nucleic acid test assays has recently been demonstrated to be higher than that found with assays designed to detect high viral titers in the plasma manufacturing sector.
STUDY DESIGN AND METHODS: Stored plasma aliquots from 5020 donations collected between 2000 and 2003 at seven US blood centers were tested. Testing was performed with a real-time B19 DNA polymerase chain reaction (PCR; TaqMan, Applied Biosystems) assay with a 50 percent limit of detection (LOD) of 1.6 IU per mL (95% confidence interval [CI], 1.2-2.1 IU/mL) and a 95 percent LOD of 16.5 IU per mL (95% CI, 10.6-33.9 IU/mL). Confirmation and quantitation of B19 DNA was accomplished by retesting of two additional subaliquots. Confirmed-positive specimens were tested for the presence of anti-B19 immunoglobulin M (IgM) and IgG with FDA-licensed assays.
RESULTS: B19 DNA prevalence was 0.88 percent (95% CI, 0.64%-1.2%). Among the 23 donations with B19 DNA titers of at least 20 IU per mL, the median DNA concentration was 105 IU per mL with an interquartile range of 42 to 481 IU per mL; the highest value was 1869 IU per mL. All B19 DNA–positive donations were positive for the presence of IgG and 10 (23%) were also positive for the presence of IgM; IgM seropositivity was associated with increasing DNA levels ($p = 0.0013$).
CONCLUSION: Low-level B19 DNA was detected in nearly 1 percent of donations. The 23 percent of DNA-positive donations with both IgM and IgG B19 antibody most likely represent acute resolving infection, whereas those with IgG but no IgM are most consistent with a more chronic and possibly persistent phase of B19 infection.

Parvovirus B19 infection (also known as human erythrovirus and referred to as B19 in this report) has been well documented to be transmitted by transfusion of plasma derivatives.[1-3] There are only rare case reports, however, of B19 transmission by transfusion of blood components, and two small studies that attempted to assess such transmission systematically did not demonstrate any symptomatic infection.[4-9] To date, there have been no large-scale linked transfusion transmission studies with sufficient statistical power to allow for a systematic calculation of the per unit or per recipient risk of acquiring asymptomatic or symptomatic infection after transfusion of a B19-viremic blood component.

In the plasma derivative setting, B19 transfusion transmission has not been reported when the plasma B19 DNA concentration was less than 10^3 international units

ABBREVIATIONS: C_T = cycle threshold; LOD = limit of detection.

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(IU) per mL.[1] It is unknown if recipients of pooled plasma products with low B19 viral titers are protected due to the neutralizing effect of B19 antibody from other units in the plasma pool, the low B19 viral titer, or a combination of both.[10] Although it has been assumed that single-unit blood components with low B19 DNA titers should, similarly, be noninfectious, this remains speculative because the mechanism of protection in the pooled plasma setting has not been established and may not apply to single-unit transfusions.

Newer information suggests that the potential for recipients to be exposed to low titers of B19 DNA from blood component transfusion is greater than previously thought. Through the use of sensitive nucleic acid test (NAT) assays, two sets of investigators have found that the prevalence of B19 DNA in donor plasma ranges from 0.5 to 0.9 percent.[11,12] Furthermore, it is now known that B19 DNA may persist in plasma at low concentration for several years in healthy individuals who could make repeat blood donations during this viremic interval.[13-16]

From 2000 through 2003, NHLBI and CDC established the Retrovirus Epidemiology Donor Study Allogeneic Donor and Recipient (RADAR) repository as a powerful tool to investigate possible transfusion-transmitted infections.[17] Our primary aim with regard to B19 infection was to use this linked donor and recipient repository to evaluate whether donations with low B19 DNA levels transmit infection. Such a transfusion transmission study would only be feasible, however, if a B19 NAT assay with appropriate performance characteristics (e.g., sensitivity, specificity, throughput) was available and if the prevalence of plasma B19 DNA in the donor population and the number of susceptible recipients were of sufficient magnitude to allow for significant conclusions to be drawn.

The primary aim of this report is to present the results of these initial investigations, which include development of a highly sensitive polymerase chain reaction (PCR) screening assay and estimation of the prevalence of plasma B19 DNA in donations represented in the RADAR repository. Our secondary aims were to evaluate the association of quantitative DNA levels with immunoglobulin M (IgM) and IgG antibody status and determine the demographic characteristics of B19 DNA–positive donors.

MATERIALS AND METHODS

Selection of repository specimens

The RADAR repository was established from 2000 through 2003 through participation of blood centers and selected hospitals at seven geographically dispersed locations throughout the United States.[17] This donor and recipient repository contains pretransfusion (or peritransfusion) specimens and follow-up specimens (collected at an interval of 6-12 months) from 3,575 enrolled recipients. The repository also contains 13,201 donation specimens given by 12,408 distinct donors that were transfused to these RADAR recipients. This portion of the repository is referred to as the linked donor-recipient repository. In addition, there is a supplementary repository of 99,906 donation specimens (contributed by 84,339 donors) from donations that were not transfused to enrolled RADAR recipients.

As previously reported, transfusion transmission studies with the RADAR-linked repository should usually only be considered if the donor prevalence of an agent is at least 0.05 percent.[17] For this study, based on some of the conservative estimates of donor B19 viremia in the literature, we determined that testing of approximately 5,000 specimens would allow us to be 95 percent confident that the prevalence of viremia in the donor population was at least 0.05 percent. Thus, 5,200 specimens (allowing for failed runs) were selected from the repository of unlinked community whole-blood and apheresis donations for B19 DNA PCR testing. A stratified sampling procedure was used to select these specimens so that they would have similar demographic, temporal, and geographic characteristics to the 13,198 community whole-blood and apheresis donation samples in the linked repository, thereby allowing for later extrapolation of the prevalence results to donations in the linked repository. The sampling scheme controlled for frequency of donations per donor, blood center where donation was given, and year and month of donation, in that order. This stratification also ensured that the distributions of other important variables, for example, age at time of donation, first-time or repeat status, and race/ethnicity were similar between the 5,200 sampled unlinked donations and the 13,198 donation samples in the linked repository. The similarity of the sampled supplementary repository subset and the linked donations was verified after the sample was selected.

A 1.75-mL frozen plasma tube for each selected donation was accessed from the repository by personnel at the long-term storage facility (SeraCare BioServices, Gaithersburg, MD). Each specimen was aliquoted into three 0.5-mL subaliquots (one for B19 DNA screening and two for B19 DNA confirmation and quantitation) and one 0.25-mL aliquot (for antibody testing) with rigorous precautions to minimize the possibility of cross-sample contamination.

PCR assay development, validation, and performance characteristics

The B19 DNA assay used in this study was originally developed by Chiron Corp. (Emeryville, CA) and subsequently refined through a collaboration between Chiron and Blood Systems Research Institute (San Francisco, CA).[18] The assay format includes a magnetic-bead B19 DNA capture step followed by a real-time PCR assay that targets the VP1 region of the B19 genome. An internal control, sharing homologous primer region sequences but with a different internal probe binding sequence as the viral

target, is included in each assay tube. B19 DNA target and the internal control nucleic acid are amplified by the same primer pair but detected and distinguished by fluorophore-tagged sequence-specific probes. Five-hundred microliters of frozen plasma, thawed at room temperature, was vortexed and centrifuged briefly before the addition of lysis buffer, poly(T)-coupled magnetic beads (Seradyn, Indianapolis, IN), four viral capture primers (VSCP1, VSCP4, VSCP5, and VSCP7) with poly(A) tail, and 20 copies of internal control. The preparation was vortexed for 10 seconds and incubated in a 60°C water bath for 20 minutes, followed by incubation at room temperature for 15 minutes. The tubes were placed on a magnetic base for 10 minutes before the liquid was vacuum-aspirated. The beads were washed once with 1 mL of wash buffer (Procleix, Gen-Probe, San Diego, CA) and twice with another wash buffer (Chiron Novartis, Emeryville, CA).

All captured target DNA from the 0.5-mL input plasma and captured spiked internal control were subjected to amplification in a single PCR procedure and amplification and detection occurred in a 96-well optical plate with dual-plexed TaqMan PCR technology. TaqMan 1000 Rxn PCR core reagents were purchased from Applied Biosystems (Foster City, CA). The PCR mix was prepared by mixing 10 µL of Buffer A; 1 µL of the enzyme uracil-*N*-glycosylase (Amperase [Roche Diagnostics, Indianapolis, IN], which reduces contamination by degrading dUTP-containing amplicons from prior amplification reactions); 20 µL of $MgCl_2$; 10 µL of dATP, dCTP, dGTP, and dUTP; 0.5 µL of AmpliTaq Gold; 56 µL of sterilized water; 0.9 µL each of two amplification primers at 100 pmol per µL (VSCP8, VSCP9); and 0.25 µL of each of the two probes at 100 pmol per µL (VSCP10, VB-TAM) per sample. One-hundred microliters of the mix was added to each sample instead of the manufacturer-suggested 50 µL per sample. PCR was performed with 50 cycles of 95°C for 15 seconds and 60°C for 1 minute, after the initial Amperase (50°C for 2 min) and AmpliTaq Gold activation (95°C for 10 min). The DNA was amplified and detected with a real-time PCR system (ABI 7500, Applied Biosystems).

Features of the assay system that minimize risk of specimen-to-specimen cross-contamination of plasma or "carryover" amplicon contamination include single-tube magnetic bead target-capture and DNA purification with the Chiron/Gen-Probe–enhanced semiautomated system, single-tube amplification, and real-time monitoring of fluorescent probe binding to amplicon products with no subsequent manipulation of reaction wells; use of dUTP and UNG in each assay to destroy previous B19 amplicons before amplification; and single-use disposable reaction tubes and plates. Segregated laboratories were used for sample accessioning and preparation, preamplification target-capture, and real-time PCR.

Preliminary assay development work used a series of dilutions of the CBER parvovirus B19 DNA standard to determine where to set the assay cutoff as well as to estimate the resultant assay analytic sensitivity. Figure 1 shows box and whisker plots of testing results for 30 replicates at each of four dilutions (30, 15, 7.5, and 3.75 IU/mL). Based on these studies, the assay cutoff was established as follows: a specimen was classified as reactive if a signal was detected at not more than 40 cycles (cycle threshold [C_T] ≤ 40), indeterminate if C_T was more than 40 but not more than 45, and negative if there was no signal detected or if a C_T was more than 45. An apparent negative result was interpreted as invalid if the C_T of the internal control was more than 45.

Because the chosen assay cutoff was designed to maximize assay sensitivity, an algorithm was developed for final test interpretation so as to avoid classifying nonspecific reactivity on a single assay run as a confirmed-positive result (see Fig. 2). All initially positive, initially indeterminate, and invalid specimens were retested in duplicate with two separate 0.5-mL subaliquots on plates that included quantitative run standards. This testing

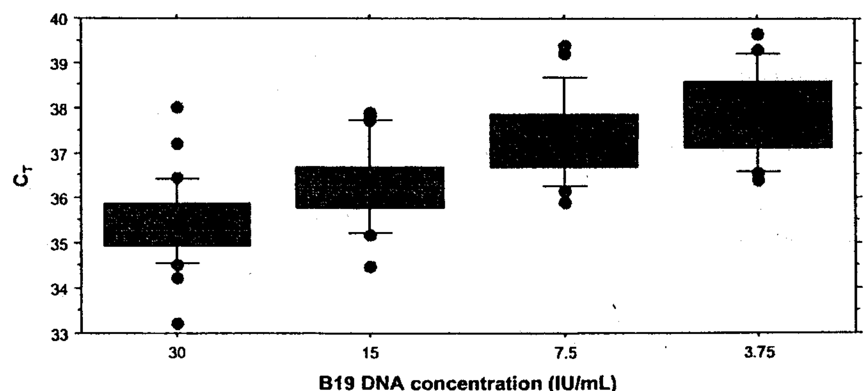

Fig. 1. Assay validation and selection of assay cutoff based on replicate testing of dilutions of the CBER parvovirus B19 DNA standard. The input per amplification was 0.5 mL. Twofold dilutions of CBER-validated B19 DNA standard were run with 30 replicates at each concentration. The standard was diluted with pooled plasma negative for B19 DNA and B19 antibody. The y-axis represents PCR C_T. The x-axis represents B19 DNA concentration per milliliter of plasma. The top and bottom whiskers on the box plot represent the 90th and 10th percentiles, respectively. The top of the box represents the 75th percentile and the bottom of the box represents the 25th percentile. The line inside the box represents the median. Negative plasma aliquots were also tested (data not shown). Of 204 B19-negative plasma aliquots, 203 yielded negative assay results (C_T > 40). One negative control sample amplified at 36.30 C_T.

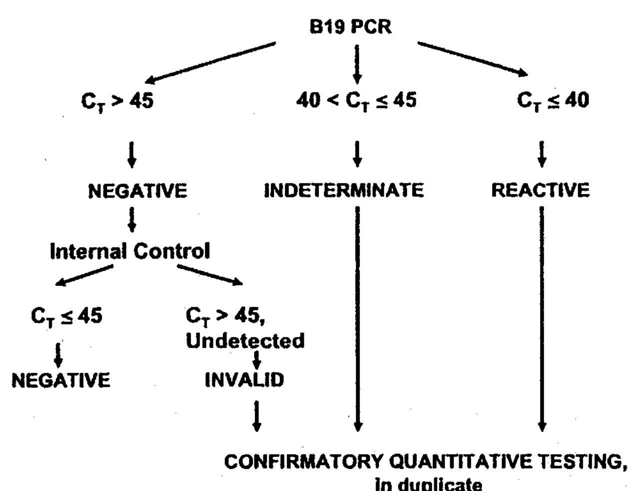

Fig. 2. B19 DNA testing algorithm.

served both as confirmation and as quantitation. The final interpretation of the qualitative PCR assay was based on the results of the three individual assays (i.e., the initial screening assay and the duplicate repeat assays). Specimens were classified as B19 DNA–positive if at least two of three tests showed reactivity at a C_T of not more than 40 cycles and indeterminate if at least two tests showed reactivity at a C_T of not more than 45, with one or both of these C_T values more than 40, and negative if both of the duplicate repeat assays were negative.

For determining DNA concentration, quantitative run standards were placed on each plate in duplicate. For confirmation and quantitation of initially reactive specimens, standards from 1000 to 31.25 IU per mL were tested in twofold dilutions. For repeat testing of indeterminate and invalid specimens, standards from 125 to 31.25 IU per mL were tested in twofold dilutions to prevent any cross-contamination of the specimens from high-titer standards. Quantitative results were determined by comparing the C_T of the specimen to the C_T of the known standards on the same test run. The assigned quantitative value for each specimen was the average of the two duplicate quantitative assays (including zero for a negative test result). Specimens with low C_T (C_T < 30) were diluted 1:10 and 1:100 and then run in triplicate at each dilution, and the quantitative result was the mean of the three test results at the most appropriate dilution.

Analysis of additional replicates of the CBER standard (30-60 replicates performed on twofold serial dilutions with concentrations of 30, 15, 7.5, 3.75, 1.88, and 0.94 IU/mL) established that the 50 percent limit of detection (LOD) of the assay was 1.6 IU per mL (95% confidence interval [CI], 1.2-2.1 IU/mL), and the 95 percent LOD was 16.5 IU per mL (95% CI, 10.6-33.9 IU/mL). To allow for the possibility that quantitation might not be precise at the lower limits of detection, we categorized all specimens with quantitative values of 0 to less than 20 IU per mL as having a value of less than 20 IU per mL.

PCR testing of study specimens

Initial B19 DNA testing was performed in singlicate with one 0.5-mL plasma aliquot. Testing was performed in 96-well microtiter plates. Each plate contained two known positive, two blinded negative, and two blinded positive controls as well as up to 90 study specimens. All positive controls were prepared by the testing laboratory (BSRI) from the CBER parvovirus B19 DNA standard and were diluted to contain 100 IU per mL B19 DNA. The known controls were introduced into each test batch by the testing laboratory whereas the blinded controls were introduced into each specimen batch by the repository facility. Runs were considered valid if at least one of the two known positive and one of the two known negative controls gave a valid, expected result. Figure 3 shows the high consistency of assay performance on the known and blinded positive control specimens for 56 screening test runs based on C_T; the C_T for the known controls (Fig. 3A) was 33.36 ± 2.96 and the C_T for the blinded controls at the same concentration was 34.09 ± 2.71 (Fig. 3B). All positive controls reacted with the exception of 5 of 112 known positive controls with invalid results and 1 of 112 blinded positive controls with a false-negative result. In addition, 110 of 112 negative controls were negative, 1 gave an invalid result, and 1 gave an indeterminate result.

All initially positive, indeterminate, and invalid specimens were rerun in duplicate with two separate subaliquots on plates that included quantitative run standards. Because of limitations of specimen volume, this testing served both as confirmation and as quantitation (see above).

B19 antibody testing

All confirmed B19 DNA–positive and indeterminate donations were tested for the presence of B19 IgG and IgM antibodies against a recombinant VP2 protein with FDA-licensed test kits (Biotrin, Dublin, Ireland). Testing was performed in singlicate with the 0.25-mL subaliquot. 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.

Additionally, to determine IgG and IgM prevalence in B19 DNA–negative donations, we first randomly selected a subset of 520 donation specimens from the 5200 donations that had been selected for PCR testing (see above). This sampling occurred before obtaining the PCR results on the 5200 donations. IgG antibody testing was performed on 505 of the 520 donations, 501 of which were subsequently found to be B19 DNA–negative. For IgM antibody, due to kit availability issues, a random subset of

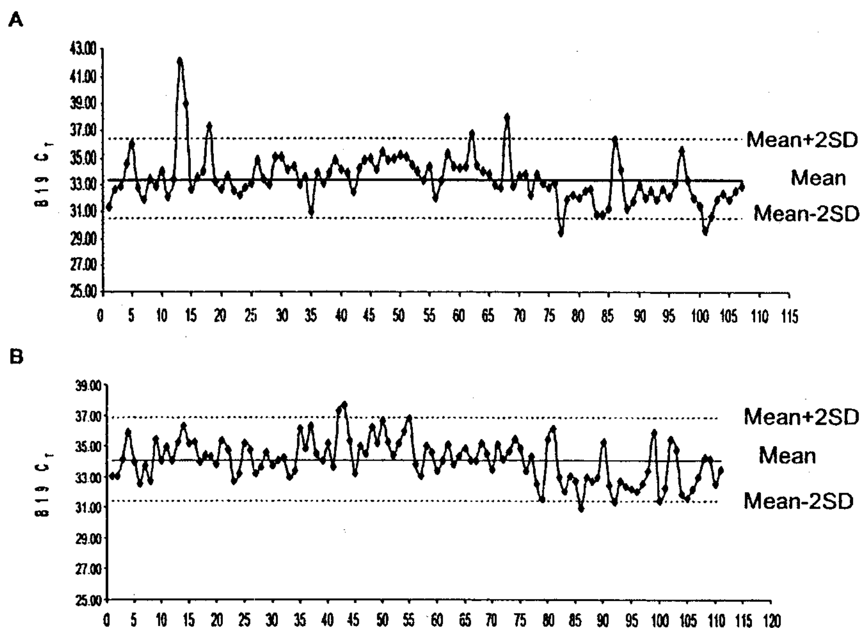

Fig. 3. (A) Control chart: 100 IU per mL known controls. Two known positive controls were included per run. The data plotted were controls for 56 plates, which includes 107 valid results and excludes 5 invalid results (C_T mean ± 2SD = 33.36 ± 2.96). (B) Control chart: 100 IU per mL blinded controls. Two blinded positive controls were included per run along with two blinded negative controls (data not shown). The data plotted were positive controls for 56 plates, which includes 111 valid results and one false-negative result (C_T mean ± 2SD = 34.09 ± 2.71).

194 of 366 of these specimens identified as IgG-positive were tested.

Demographics

The following information was available for each donation in the RADAR repository: donor identification number, age at time of donation (categorized as ≤25, 26-35, 36-45, 46-55, 56-65, ≥66), sex, race/ethnicity (Asian, black non-Hispanic, Hispanic, white non-Hispanic, other non-Hispanic), first-time or repeat donor status, education level (<high school, high school degree, some college education, college degree, graduate or professional degree), history of transfusion, the center at which the donation was collected, and date of donation (categorized by calendar year of donation or by season, i.e., occurring in the winter, spring, summer, and fall).

Statistical analysis

We calculated the proportion of donations that were confirmed positive by PCR with associated 95 percent CI as well as the prevalence of IgM and IgG (and their 95% CI) in DNA-positive and DNA-negative donations. We evaluated whether the prevalence of IgM in DNA-positive donations varied as a function of B19 DNA level (categorized as <20, 20 to $<10^2$, 10^2 to $<10^3$, and 10^3 to $<10^4$ IU/mL) by conducting a Fisher's exact test (SAS/STAT 9.1, 2004, SAS Institute, Inc.). We determined the 50 and 95 percent LOD of our PCR assay with associated 95 percent CI by probit analysis with PC SAS Version 8.2.

We compared the distribution of demographic characteristics between donation groups (i.e., DNA-positive and DNA-negative donations) with chi-square statistics or, for small cell sizes, either the Fisher's exact test (SAS/STAT 9.1, SAS Institute, Inc.) or the Fisher-Freeman-Halton test (StatXact Version 6, 2004, Cytel Software Corp., Cambridge, MA). This latter test is a generalization of the Fisher's exact test for 2 by 2, to an r-by-c contingency table.

RESULTS

Of the 5200 specimens originally selected for DNA testing, screening results were obtained for 5020. Results were not obtained for 180 specimens that were part of two runs which failed due to equipment problems. There were 113 initially reactive specimens (2.25%), 26 initially indeterminate specimens (0.52%), 56 initially invalid specimens (1.12%), and 4825 negative specimens. After retesting, 43 of the initially reactive specimens confirmed as positive, 2 were reclassified as indeterminate, and 68 were reclassified as negative. For the indeterminate specimens, 1 was reclassified as positive, 22 remained indeterminate, and 3 became negative. All initially invalid specimens retested as negative.

Summarizing the screening and retesting results, we found that 44 specimens (0.88%) were DNA-positive, 5 (0.10%) were indeterminate, and 4971 (99.02%) were negative. In 35 (80%) of the confirmed-positive specimens, all three tested replicates reacted in the PCR assay. DNA prevalence was 0.88 percent with a 95 percent CI of 0.64 to 1.2 percent.

Figure 4 shows that the percentage of confirmed-positive specimens was inversely related to the C_T value obtained on the initial screening test run. Specimens that initially reacted at a C_T value of less than 37 were confirmed as positive 86 percent of the time, whereas specimens with a C_T value of between 37 and 40 were confirmed 16 percent of the time.

Table 1 presents the quantitative DNA levels grouped into four categories as well as the antibody status of the 44 B19 DNA confirmed–positive donations. The median DNA level for all of our confirmed-positive donors was 22.75 IU

per mL. Twenty-one of 44 specimens had DNA levels of less than 20 IU per mL (conservatively determined to be the lower limit of quantitation of the assay), and if we consider only those donors who had DNA levels of more than 20 IU per mL, then the median DNA level for these 23 donors was 105 IU per mL (interquartile range, 42-481 IU/mL), with the highest value being 1869 IU per mL. Specimens with reactivity on two of the three replicates had lower DNA levels than specimens reactive on all three replicates (data not shown).

All B19 DNA confirmed-positive donations had detectable B19 IgG antibody, whereas in the control group of 501 PCR-negative donors, IgG was present in 73 percent (95% CI, 68%-77%). IgM antibody was detected in 10 B19 DNA confirmed-positive donations and was assigned an equivocal status in 2 additional cases. IgM seropositivity was associated with increasing DNA concentration ($p = 0.0013$). The median DNA level for the 10 IgM-positive donations was 297 IU per mL, and all three donors with B19 DNA titers of more than 10^3 IU per mL were IgM-positive. IgM was not detected in any of 194 DNA-negative, IgG-positive donors (95% CI, 0.00%-1.88%).

Donors who were not more than 45 years old were more likely to be viremic than donors older than 45 years:

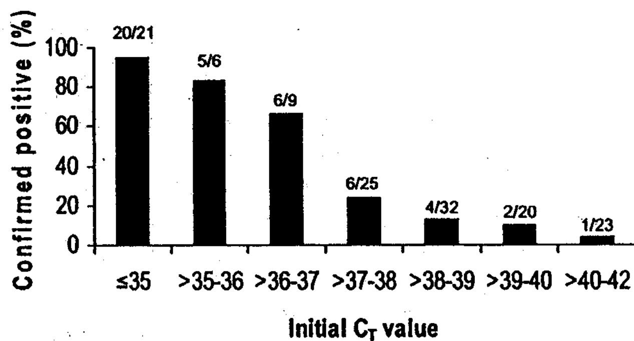

Fig. 4. Confirmation of B19 DNA reactivity relative to the C_T obtained on initial PCR screening. Confirmation of all 136 initially reactive specimens. There were no initially reactive specimens with C_T between 42 and 45 cycles. All 4884 specimens with a C_T value of more than 45 on the initial test run were classified as negative.

1.24, 1.30, and 1.41 percent of donors not more than 25, 26 to 35, and 36 to 45 years, respectively, were viremic compared to 0.23, 0.37, and 0.00 percent of donors 46 to 55, 56 to 65, and more than 65 years old ($p = 0.0008$). Further, 1.85 percent of first-time donors were viremic compared to 0.66 percent of repeat donors ($p = 0.007$). Although DNA prevalence estimates appeared to be higher for spring (1.14%) and summer (1.18%) donations than for fall (0.44%) and winter (0.67%) donations, these differences did not achieve significance ($p = 0.09$). There was no significant association with geographic region (based on center where the donation was given), calendar year of donation, sex, race/ethnicity, education level, or transfusion history.

DISCUSSION

In this study we applied a highly sensitive B19 DNA assay to 5020 individual donations to determine the prevalence of plasma B19 DNA in donors from seven different geographic areas of the United States using specimens collected over a 4-year interval. We believe this to be the largest such study performed on individual donations rather than on large pools of donations, as is routine in the plasma manufacturing sector.

We found that the prevalence of B19 DNA in plasma was 0.88 percent with a 95 percent CI of 0.64 to 1.2 percent. Our data can be compared with several recent European studies that used somewhat less sensitive B19 DNA assays. Thomas and coworkers[11] tested 16,859 Belgian blood donors in pools of 60 donations with an assay with 95 percent LOD of 96.6 IU per mL and found a B19 DNA prevalence of 0.16 percent. Candotti and colleagues[12] tested 1,000 UK whole-blood and platelet donors in minipools of 10 donation specimens and found a prevalence of 0.9 percent with a nested PCR with a 95 percent LOD of 25 IU per mL; this prevalence was very similar to that found in our study. Plentz and colleagues[8] found a 0.7 percent prevalence with an assay with a 50 percent LOD of 60 to 80 copies per mL when retrospectively testing 1,806 blood products transfused in a hematology ward. In contrast, studies reported in the plasma manufacturing sector have reported much lower prevalence, ranging from 0.008 to 0.04 percent; it is notable that these

TABLE 1. Quantitative PCR and antibody results on confirmed-positive specimens*

Viral load (IU/mL)	Number of confirmed-positive specimens	IgM-positive and IgG-positive	IgM-equivocal and IgG-positive	IgG-positive only
<20	21	1 (5)	1 (5)	19 (90)
20 to <10^2	11	2 (18)	0 (0)	9 (82)
10^2 to <10^3	9	4 (44)	1 (11)	4 (44)
10^3 to <10^4	3	3 (100)	0 (0)	0 (0)
Total	44	10 (23)	2 (4)	32 (73)

* Data are reported as number (%).

studies used NAT assays that were designed to lack sensitivity so as to only detect units from donors in the stage of acute viremia with DNA concentrations of more than 10^5 or 10^6 IU per mL.[19-21]

The generally accepted understanding of the natural history of B19 infection in immunocompetent individuals such as blood donors states that viremia occurs approximately 1 week after infection and persists in high titer for approximately 5 days. With the development of IgM antibody at approximately 12 days after infection (followed within days by IgG antibody), viremia levels drop precipitously and viremia usually disappears within weeks.[22,23] IgM antibody becomes undetectable after several months (although this precise duration is unknown) but IgG persists long term and is thought to convey immunity to reinfection. As a variation of this usual natural history, plasma viremia may persist for more than 6 months to several years in some cases, and recent data suggest that B19 may persist in other tissue sites (e.g., skin, synovia) for a much longer period of time in a significant percentage of individuals.[13-16,24]

The antibody findings in our study can be used to assess the stage of viremia that we detected in our B19 DNA–positive donors. We did not detect any B19 DNA–positive donors who lacked both IgM and IgG antibody nor did we detect any donors with a DNA concentration at or above 10^5 or 10^6 IU per mL, which would be characteristic of the several-day interval after infection. Given the low rate of detection of high-titer DNA in previous studies, it is not surprising that with the testing of 5020 donations in this study, we did not detect any such donations. We also did not detect any donors in the short window period where IgM antibody is present but IgG is absent. We detected 10 donors who were positive for the presence of IgM and IgG (23% of the 44 B19 DNA–positive donors) and an additional 2 who were IgM-equivocal. These donors were probably in a relatively early stage of infection, that is, within the first several months of acquiring infection. Consistent with the known natural history of B19 infection, the IgM-positive, DNA-positive donors had higher DNA levels than the IgM-negative, DNA-positive donors. We detected 32 DNA-positive donors who were IgG-positive only: 28 of these had DNA levels of less than 10^2 IU per mL; the median DNA level in these donors (as for all 44 DNA-positive donors) was lower than that previously reported by other investigators. We believe either that these DNA-positive, IgG-positive donors were at the tail end of resolving their B19 viremia or that some of these donors may have had very-low-titer B19 DNA that persisted for longer than predicted by the standard natural history model. Future longitudinal studies will be needed to distinguish these possibilities. The 73 percent prevalence of B19 IgG seropositivity and the lack of IgM antibody in our B19 DNA–negative control donors were consistent with reports in other donor cohorts.[11,12,25]

B19 infections are known to occur with a spring and summer preponderance and to vary in annual frequency in cycles that span several years.[25] In our study, the B19 DNA prevalence was higher in spring and summer donations, but did not achieve significance. This may be due to limitations in sample size or alternatively to our use of a highly sensitive NAT assay that may have allowed us to detect B19 DNA–positive donors for a relatively long period of time after acquisition of infection, thereby masking the expected temporal findings. The significant association with younger age (<age 46) may correlate with an increased likelihood of possible B19 exposure in young adults from contact with young children or with a lower degree of susceptibility among previously infected older adults. First-time donors showed higher prevalence of viremia than repeat donors, which may in part reflect the younger age distribution of first-time donors or may be due to other unexplained factors as has been seen with other infectious agents (e.g., human immunodeficiency virus, hepatitis C virus) in the donor population.[26]

The real-time B19 TaqMan PCR assay used in this study gave reproducible results on known standards, had a low failed run rate, gave a low rate of invalid specimens due to internal control failure, and showed no evidence of sample cross-contamination. Furthermore, the assay had a high analytic sensitivity at the chosen cutoff (50% LOD, 1.6 IU/mL; 95% LOD, 16.5 IU/mL). Although our choice of a relatively high C_T cutoff introduced nonspecificity on initial testing (presumably due to nonspecific probe binding), our confirmatory algorithm minimized false-positive results by requiring a reactive result on a second aliquot subjected to the full extraction, amplification, and detection procedure before designating the donation as confirmed positive for the presence of B19 DNA.

At present, interventions for preventing B19 transfusion from blood components have not been implemented in the vast majority of developed countries, due in part to the prevailing view that blood components with low levels of B19 DNA will not transmit B19 infection. Recently several authors have cited the need for studies to verify this hypothesis.[11,12] The results of the study reported in this article have established that there is sufficient statistical power to carry out such a B19 transfusion transmission study with the real-time B19 TaqMan PCR assay to test specimens in the RADAR repository. This conclusion is based on the demonstrated donor B19 DNA prevalence of 0.88 percent combined with a sufficient number of B19-susceptible recipients in the RADAR repository; that is, we observed that 22 percent of tested RADAR recipients were B19 IgG–negative on their pretransfusion specimen (data not shown), which was similar to the 27 percent prevalence of IgG seronegativity in tested donors. We are now actively engaged in per-

forming a linked B19 transfusion transmission study using the RADAR repository.

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別紙様式第２－１

医薬品　研究報告　調査報告書

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研究報告の概要

ヒトパルボウイルスB19（B19V）は血液試料に多く認められるヒト病原体であり、主に呼吸器経路を介して伝播される。B19Vは他のパルボウイルスよりも物理化学的な処理に対する感受性が高いが、その理由は未だ明らかではない。
血漿プールは数百の血液で構成されるため、PCR法で検査すると大半の血漿プールからB19V DNAが検出され、第VIII および第IX凝固因子、ヒト血清アルブミン、静注用免疫グロブリン、筋注用免疫グロブリン、プロトロンビン複合体濃縮製剤、アンチトロンビンIIIなどで報告があるが、いずれもB19V DNAを検出したことを証明しているのであって、必ずしも感染性のあるウイルスの存在を証明しているわけではない。しかし、血漿分画製剤による伝播の報告もある。
パルボウイルスは最も安定なウイルス群に属し、物理化学的な処理の多くに抵抗性であるが、B19Vは乾熱または湿熱のほか、低pHまたは高pH、UVC照射、光化学反応により不活化できる。
本報告ではB19V不活化能について「60℃・10分」および「pH4・2時間」の2条件で評価したところ、ウイルスの感染性は低下し検出限界未満となり、既発表データと一致していた。
熱または低pHによるB19V不活化機序は、DNAを包むカプシドの分解ではなく、カプシドからのDNAの遊離によるものであることがわかったが、熱安定化剤としてクエン酸を用いるとB19V DNAはカプシドから遊離せず、感染性が維持される。

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使用上の注意記載状況・その他参考事項等

慎重投与の項
・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。］
・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。］
重要な基本的注意の項
(1)本剤の原材料となる‥［スクリーニング項目、不活化・除去工程］‥投与に際しては、次の点に十分注意すること。
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。
妊婦、産婦、授乳婦等への投与の項
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。］

報告企業の意見	今後の対応
ヒトパルボウイルスB19の不活化について「60℃・10分」および「pH4・2時間」の2条件で評価したところ、感染性は検出限界未満となり、既発表データと一致していたとの報告で、他のパルボウイルスに比べ不活化されやすいとも述べらている。 なお、弊社血漿分画製剤は最終製品において核酸増幅検査によりヒトパルボウイルスB19 DNAが陰性であることを確認している。	今後ともヒトパルボウイルスB19に関する血漿分画製剤の安全性に関する情報に留意していく。

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Molecular mechanism underlying B19 virus inactivation and comparison to other parvoviruses

Bernhard Mani, Marco Gerber, Patricia Lieby, Nicola Boschetti, Christoph Kempf, and Carlos Ros

BACKGROUND: B19 virus (B19V) is a human pathogen frequently present in blood specimens. Transmission of the virus occurs mainly via the respiratory route, but it has also been shown to occur through the administration of contaminated plasma-derived products. *Parvoviridae* are highly resistant to physicochemical treatments; however, B19V is more vulnerable than the rest of parvoviruses. The molecular mechanism governing the inactivation of B19V and the reason for its higher vulnerability remain unknown.
STUDY DESIGN AND METHODS: After inactivation of B19V by wet heat and low pH, the integrity of the viral capsid was examined by immunoprecipitation with two monoclonal antibodies directed to the N-terminal of VP1 and to a conformational epitope in VP2. The accessibility of the viral DNA was quantitatively analyzed by a hybridization-extension assay and by nuclease treatment.
RESULTS: The integrity of the viral particles was maintained during the inactivation procedure; however, the capsids became totally depleted of viral DNA. The DNA-depleted capsids, although not infectious, were able to attach to target cells. Comparison studies with other members of the *Parvoviridae* family revealed a remarkable instability of B19V DNA in its encapsidated state.
CONCLUSION: Inactivation of B19V by heat or low pH is not mediated by capsid disintegration but by the conversion of the infectious virions into DNA-depleted capsids. The high instability of the viral DNA in its encapsidated state is an exclusive feature of B19V, which explains its lower resistance to inactivation treatments.

B19 virus (B19V) is the only well documented human pathogen of the *Parvoviridae* family. The virus belongs to the genus *Erythrovirus*. In most cases, the infection is either asymptomatic or accompanied by mild nonspecific symptoms. The most common syndrome caused by B19V is an erythematous rash illness named erythema infectiosum affecting children. B19V is also the causative agent for transient aplastic crisis, which may have severe effects on patients suffering from sickle cell disease and other anemic illnesses. Chronic infections accompanied by pure red cell aplasia and anemia affect immunocompromised patients. Furthermore, B19V may cause fetal death, autoimmune diseases, and arthropathies.[1]

B19V is a widespread pathogen. The serologic evidence of a past infection is 40 to 60 percent for young adults and 80 to 100 percent for elder people.[1,2] Owing to its high prevalence, blood donations are frequently contaminated with B19V. The measured incidence of contamination depends on the sensitivity of the detection method and ranges from 0.003 percent (immunodiffusion) to 1.2 percent (polymerase chain reaction [PCR]) of blood donations examined.[3-7] Because plasma pools are constituted of hundreds of donations, B19V DNA is found in the majority of plasma pools as determined by PCR.[8-10] The contamination of plasma-derived products, such as coagulation factors VIII and IX, human serum albumin, intravenous immune globulin, intramuscularly injected

ABBREVIATIONS: B19V = B19 virus; MVM = minute virus of mice; PBSA = phosphate-buffered saline containing 1 percent bovine serum albumin; PLA_2 = phospholipase A2.

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immune globulin, prothrombin complex concentrate and antithrombin III has been reported.[8,9,11,12] Therefore, there is a risk of transmitting B19V through the administration of plasma-derived products. In these studies however, the contamination was demonstrated with the presence of B19V DNA with PCR, which does not necessarily prove the presence of infectious virus. Nevertheless, direct evidence of B19V transmission through the administration of plasma-derived products has also been shown in several case studies.[13-16] Moreover, patients that receive such medication on a regular basis show a higher prevalence of B19V-specific antibodies than control groups.[17] Altogether, the contamination of plasma-derived products indicates a potential risk of a B19V infection for the treated patient with potentially severe consequences for pregnant women and anemic and immunocompromised patients.

To achieve maximal safety for plasma-derived clinical products, pathogen safety guidelines have been established, as a result of which manufacturers must demonstrate the effective elimination of viral agents during the manufacturing process of their products. Virus elimination is demonstrated either with the relevant pathogen itself or with one or several closely related model viruses. To date, there is no convenient cell culture infectivity test for B19V. For this reason, animal parvoviruses such as bovine parvovirus, canine parvovirus, porcine parvovirus, or minute virus of mice (MVM) are often used for validation studies regarding the inactivation of B19V. Parvoviruses are among the most stable viruses and have been shown to resist many common physicochemical inactivation procedures. B19V inactivation can be achieved with dry or wet heat,[18-21] as well as with low or high pH,[22,23] UVC irradiation,[24,25] or photochemical reactions.[26] Interestingly, B19V has been found to be more readily inactivated than other parvoviruses. Whereas B19V is inactivated beyond the detection limit after 10 minutes at 60°C or after 2 hours at pH 4, canine parvovirus,[21] MVM,[27] and porcine parvovirus[18] can withstand 1 hour at 60°C without considerable inactivation. Similarly, the treatment of MVM at pH 4 for 6 hours only moderately reduces its infectivity.[23] The reason why B19V is more sensitive to inactivation than other parvoviruses is not known. Although different inactivation conditions for B19V have been described, the underlying mechanism of B19V inactivation has not yet been elucidated. It is generally assumed that the inactivation occurs through capsid disintegration because the viral genome becomes accessible to DNases.[18,27] We have shown in a recent study, however, that after mild heat treatments, the DNA from B19V and MVM can be rendered accessible without capsid disintegration.[28]

In this study we have analyzed the B19V capsid rearrangements occurring during the inactivation process. The results revealed a sequence of structural transitions preceding capsid disintegration. The critical transition, which resulted in full virus inactivation, was the dissociation of the viral DNA from the still intact capsid. Comparison studies revealed that the DNA release from intact capsids is a common feature among parvoviruses but occurs much more prematurely in B19V, explaining its lower resistance to inactivation procedures.

MATERIALS AND METHODS

Cells and viruses

Human UT7/EPO cells were propagated in RPMI 1640 supplemented with 5 percent fetal calf serum (FCS) and 2 U per mL recombinant human erythropoietin (EPO; Janssen-Cilag, Midrand, South Africa) at 37°C and 5 percent CO_2. UT7 cells were provided by A. Gröner (CSL Behring, Marburg, Germany). Two B19V-containing plasma samples (Genotype 1) were obtained from two infected individuals (S-1 and S-2) and did not contain B19V-specific immunoglobulin M or immunoglobulin G (IgG) antibodies. B19V was concentrated from infected serum by ultracentrifugation through 20 percent sucrose. The viral pellet was washed and resuspended in phosphate-buffered saline (PBS). All other parvoviruses were derived from cell culture supernatant. H-1 parvovirus was provided by C. Dinsart (German Cancer Research Center, Heidelberg, Germany). Porcine parvovirus was provided by T. Novak (CSL Behring, Marburg, Germany).

Exposure of viral particles to inactivation conditions

Viral suspensions in PBS were heat-treated in thin-wall tubes for 3 or 10 minutes in a preheated thermoblock. A probe was used to monitor the temperature of the suspension. After the temperature treatment, the samples were rapidly cooled on ice and immediately used for subsequent reactions. For pH treatments, the viral suspensions were acidified by adding MES-buffered saline until the desired pH was achieved and incubated for 2 hours at 37°C. After the treatment, the pH of the viral suspension was neutralized by dilution (1:100) into PBS or in PBS containing 1 percent BSA (PBSA). Additionally, the heat sensitivity of B19V in citrate buffer, which has been recently reported to confer heat resistance to B19V,[29] was examined. The viral suspension was diluted in citrate buffer (0.5 mol/L trisodium citrate, 0.1 mol/L NaCl, pH 7) or in PBS and exposed to heat as specified above.

Infectivity assay

Titration of B19V was performed by limited dilution in quadruplicate. UT7 cells were seeded on 96-well plates (3×10^4 per well) in RPMI, containing 2 U per mL recombinant human EPO and 5 percent FCS. Virus was diluted geometrically by the factor 10 in RPMI. An equal volume of

diluted virus was added to each well and incubated at 37°C in 5 percent CO_2. After 4 days, the cell culture volume was carefully removed and cells were fixed with a solution of ice-cold methanol:acetone (1:1, v/v) for 1 hour at 20°C. After fixation, the cells were air-dried, washed with PBSA, and incubated with a mouse antibody against B19V (1:40 diluted in PBSA, clone R92F6 IgG_1, Novocastra, Newcastle upon Tyne, UK) for 1 hour at room temperature. The cells were washed with PBSA, and as secondary antibody, a conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulins was added (1:50 dilution, DakoCytomation, Glostrup, Denmark) for 1 hour at room temperature. After final washings with PBSA, the cells were overlaid with 50 µL of glycerin:PBS solution (1:1) and examined under fluorescence microscope. The infectivity titer was calculated with the Spaerman-Kärber method.[30]

Assessment of B19V capsid integrity

After exposure to heat or low pH, the integrity of the viral capsid was examined by immunoprecipitation with two different antibodies. One antibody is directed to a VP2 conformational epitope (monoclonal antibody [MoAb] 860-55D), which exclusively recognizes capsids and not denatured proteins. Another antibody recognizes an epitope in the N-terminal of VP1 (MoAb 1418).[31] The immunoprecipitation was performed overnight at 4°C in the presence of 20 µL of protein G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.5 to 1 µg antibody in a total volume of 120 µL PBSA. The supernatant was carefully removed, and the beads were washed three times with PBSA. Immunoprecipitated viral capsids were resolved by sodium dodecyl sulfate (SDS)-10 percent polyacrylamide gel electrophoresis (PAGE). After the transfer to a polyvinylidene fluoride membrane, the blot was probed with a mouse anti-B19 VPs (1:500, US Biologicals, Swampscott, MA), followed by a horseradish peroxidase–conjugated secondary antibody (1:20,000 dilution). The viral structural proteins were visualized with a chemiluminescence system (Pierce, Rockford, IL).

Assessment of B19V DNA accessibility

Subsequent to the temperature or pH treatments, the presence of externalized viral DNA was examined by a hybridization-extension assay as previously described.[28] Briefly, a probe consisting of a virus-specific 3′-end and a virus-unrelated 5′-end was hybridized to the target viral DNA and subsequently extended with sequenase (3.25 U, USB, Cleveland, OH). The extended probe was purified with a PCR purification kit (QIAquick, Qiagen, Valencia, CA) and quantified by real-time PCR. Alternatively, the presence of externalized viral DNA was examined by the treatment of the viral suspensions with DNase I (10 U, Amersham Biosciences, Piscataway, NJ) overnight at room temperature in PBS containing 6 mmol per L $MgCl_2$. The viral DNA was purified and quantified as specified below.

Quantitative PCR

The viral DNA was quantified with a real-time PCR system (LightCycler, Roche Diagnostics, Rotkreuz, Switzerland). PCR was carried out with the FastStart DNA SYBR Green kit (Roche Diagnostics) following the manufacturer's instructions. For the detection and quantification of probe-extended DNA generated from the hybridization-extension reaction, a forward primer specific for the 5′ virus-unrelated tail of the probe and a downstream virus-specific reverse primer were used. All probes and primers used are shown in Tables 1 through 3.

Assessment of the viral DNA-capsid association

To verify whether the exposed viral DNA is still associated to the capsid or otherwise dissociated, the B19V capsids were immunoprecipitated with MoAb 860-55D as indicated above. The amount of viral capsid protein and viral DNA present in the immunoprecipitated and supernatant fractions was analyzed by SDS-PAGE and quantitative PCR, respectively.

FACS analysis

The presence of B19V on the cell surface was quantitatively analyzed by flow cytometry. UT7/EPO cells were infected with either intact or heat-inactivated B19V (100 copies/cell) under conditions allowing the binding but not the internalization of the virus (4°C). The cells were washed three times and incubated with an anti-B19V capsid MoAb (5 µg/mL, 8293, Chemicon International, Temecula, CA) at 4°C for 1 hour in PBS containing 2 percent FCS, followed by an incubation with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (5 µg/mL, A85-1, BD Biosciences, San Jose, CA) at 4°C for 1 hour. The cells were analyzed by flow cytometry with a flow cytometer (FACScan, Becton Dickinson, San Jose,

TABLE 1. Probes used for the hybridization-extension assay

Virus	5′ virus-unrelated sequence	3′ virus-specific sequence
B19V	CGATCCGACTCACACCTGGACC........	CCGCCTTATGCAAATG
BPV	GGGCGAAGAACGGTGGATTAA.........	CGAGGACAGGTGGACC
CPV	GGGCGAAGAACGGTGGATTAA.........	GCGGTTTGTGTGTTTA
H-1	CCACAGAGGTCCAAGCACGCA.........	AGCGGTTCAGAGAGTT
MVM	GGGGATGCGGGGAGTGTACGGGC.......	GATAAGCGGTTCAGGG
PPV	AGGCGGTTCATGGGTGGATAG.........	GTTGCTTACTTCAGTT

TABLE 2. Primers used for PCR after the hybridization-extension assay

Virus	Forward primer	Reverse primer
B19V	CGATCCGACTCACACCTGGACC	CCCCGGTAAGGTCAAGCTTAGAAGC
BPV	GGGCGAAGAACGGTGGATTAA	CCCCCACATAGTTCATAGAAGCCT
CPV	GGGCGAAGAACGGTGGATTAA	TCCATTGCTGTTTGTGCTCCTGTA
H-1	CCACAGAGGTCCAAGCACGCA	CCGCCCCTCGTTGTAGAGACTTC
MVM	GGGGATGCGGGGAGTGTACGGGC	CCAACCATCTGATCCAGTAAACAT
PPV	AGGCGGTTCATGGGTGGATAG	CCGTTTTGTGAGGCTCTCGATT

TABLE 3. Primers used for B19V genome detection

Forward primer	Reverse primer
TGGGGCAGCATGTGTTAAA	CACAGGTACTCCAGGCACAG

TABLE 4. Effect of temperature and low-pH treatments on B19V infectivity

	S-1*	S-2
Stock	4.75†	6
pH 7.4‡	4.85	5.35
pH 4	≤2.48 ≥2.37§	≤2.48 ≥2.87
37°C	4.1	5.1
60°C	≤2.48 ≥1.62	≤2.48 ≥2.62

* S-1 and S-2 are serum samples of two infected individuals.
† Titers are given in log $TCID_{50}$ per mL.
‡ pH and temperature treatments for 2 hours and 10 minutes, respectively.
§ Reduction of infectivity.

CA). Data acquisition and analysis were conducted with software (CellQuest Pro, BD Biosciences). The percentage of cells having B19V on their surface is indicated in the upper right quadrant of each panel.

RESULTS

B19V inactivation by heat and low-pH treatments

Two different conditions, 60°C for 10 minutes and pH 4 for 2 hours, were evaluated for their capacity to inactivate B19V. After these treatments, an immunofluorescence infectivity assay was performed as described above. The applied heat or low-pH treatments resulted in the reduction of the virus infectivity beyond the detection limit (Table 4). These results are consistent with previous data on the inactivation of B19V.[18,21,23]

B19V inactivation by heat or low pH is not caused by capsid disintegration

Subsequent to the inactivation treatments by heat and low pH, the integrity of the viral capsid was examined. Viruses were immunoprecipitated with MoAb 860-55D against a VP2 conformational epitope, which recognizes only capsids.[31] The results showed that the inactivating heat treatments did not cause capsid disassembly (Fig. 1A). The capsid integrity was also examined with an antibody specific to N-VP1. As shown in Fig. 1A, after heat inactivation of B19V, VP2 could be immunoprecipitated with the antibody directed to N-VP1. Capsid disintegration was only observed increasing the incubation times at 60°C (Fig. 1B) or increasing the temperature above 60°C (Fig. 1C). As expected, treatments at 85°C resulted in the complete destruction of the viral capsids.

Similarly to the temperature treatment, inactivation of B19V by low-pH treatment was not caused by capsid disintegration. As shown in Fig. 1D, viral capsids remained assembled after exposure for 2 hours at pH 4. Moreover, exposure to more severe acidic conditions (pH 3) did not cause capsid disintegration.

B19V inactivation by heat or low pH is due to the release of the viral DNA

After the heat and low-pH inactivation treatments, the accessibility of the viral DNA was examined with a hybridization-extension assay, as described above. The results showed that while the viral capsid remained assembled, the viral genome, however, became fully accessible. The amount of accessible viral DNA was similar to that detected after complete disintegration of the viral capsids at 85°C (Figs. 2A, 2B).

To determine whether the DNA that had become accessible by the inactivation treatments was still associated with the virus capsid or otherwise dissociated, viruses were immunoprecipitated with the MoAb against capsids, and the DNA content in the supernatant and immunoprecipitated fractions was determined with quantitative PCR. As expected, in the untreated virus samples, all the viral DNA was immunoprecipitated and only a minor amount of DNA was detectable in the supernatant. Exposure of viruses to the temperature of 60°C or higher, however, resulted in total release of the viral DNA from the capsids (Fig. 2C). The same results were obtained after inactivation at pH 4 for 2 hours (Fig. 2D), indicating that the inactivation mechanism of B19V by heat or low-pH treatments was similarly caused by the conversion of the infectious DNA-containing virions into noninfectious empty capsids.

B19V DNA is not externalized and the infectivity is preserved when using citrate as thermostabilizer

It has been recently reported that in the presence of citrate, B19V becomes resistant to inactivation by pasteurization. Citrate is used as a protein stabilizer in the preparation of some plasma-derived products.[29] The mechanism by which the presence of citrate considerably

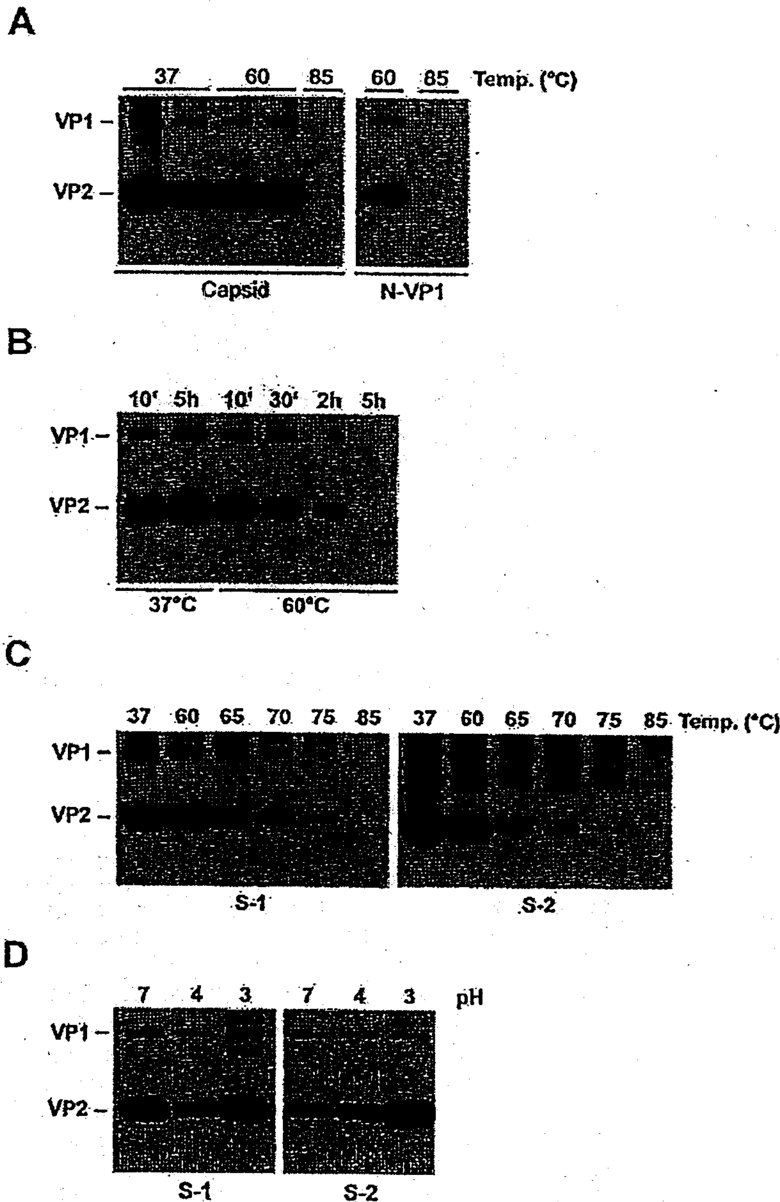

Fig. 1. Effect of inactivation by heat or low pH on B19V capsid integrity. After the exposure of B19V to different conditions, the intact capsids were immunoprecipitated and analyzed by Western blot. The immunoprecipitation was performed with an antibody directed to a VP2 conformational epitope (MoAb 860-55D), except for the right section in A, where an antibody recognizing an epitope in the N-terminal of VP1 (MoAb 1418) was used.[31] The immunoprecipitations were performed after exposure to (A) 60°C for 10 minutes, (B) increasing incubation times at 60°C, (C) increasing incubation temperatures, and (D) after exposure to low pH.

increases the heat resistance of B19V remains unknown. We have examined and compared the heat sensitivity of B19V in PBS and in a buffer containing citrate, as specified under Materials and Methods. The results confirmed that although the virus was fully inactivated in PBS, the presence of citrate conferred heat resistance and the virus could not be inactivated (data not shown). As expected, the viral DNA became fully accessible after the heat treatment of B19V in PBS but was not externalized in the presence of citrate (Fig. 3).

The inactivated DNA-depleted capsids preserve their capacity to bind cells

The capacity of the heat-inactivated B19V particles to bind the target cells was tested. The same amount of inactivated and infectious B19V was added to UT7 cells under conditions that allowed only viral binding and not internalization (4°C). Subsequently, flow cytometry analysis was performed with a B19V capsid proteins antibody as described above. The results revealed that the heat-inactivated and the infectious B19V bound to UT7 cells with a similar efficiency (Fig. 4).

B19V shows a unique DNA externalization pattern among parvoviruses

B19V is more readily inactivated than other parvoviruses. To understand the reason for this difference, the externalization of the B19V DNA was compared to that of other parvoviruses. B19V, bovine parvovirus, canine parvovirus, H1, MVM, and porcine parvovirus were exposed to increasing temperatures for 3 minutes, and the amount of accessible DNA was determined with the hybridization-extension assay. The rate of externalization was remarkably similar among all the examined viruses except for B19V (Fig. 5). At 50°C, approximately 40 percent of the B19V virions externalized their DNA, whereas barely any externalized DNA could be detected in the case of the other par-

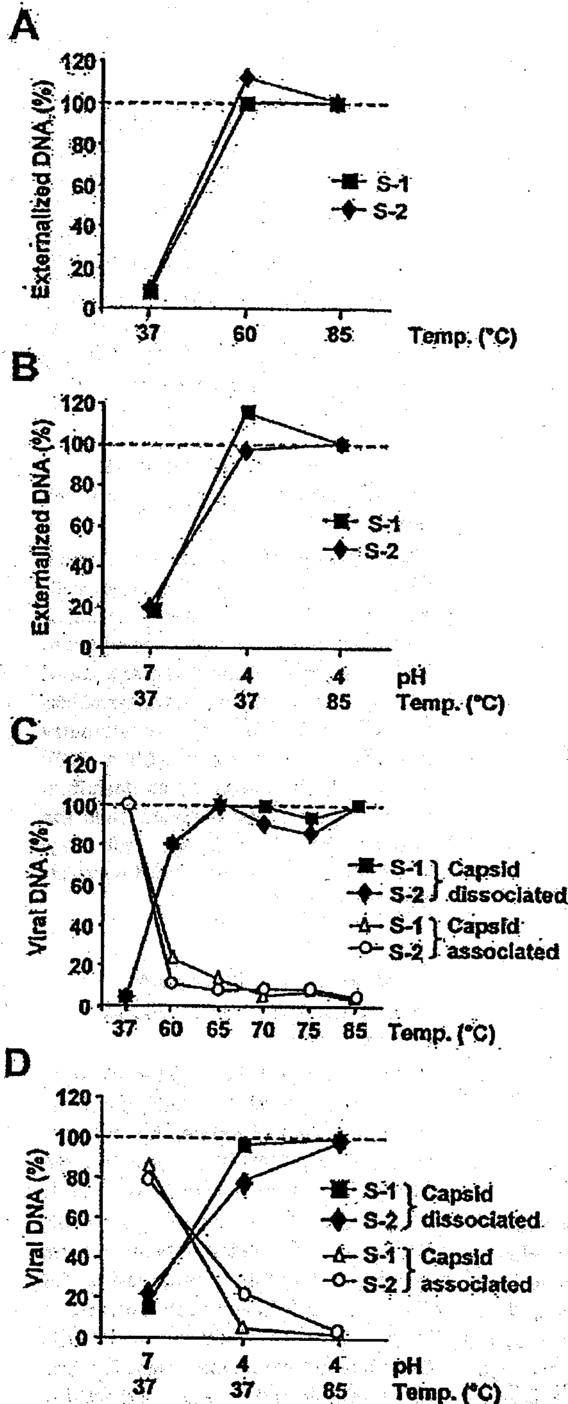

Fig. 2. Effect of inactivation by heat or low pH on B19V DNA accessibility and release. (A, B) Effect of inactivation on B19V DNA accessibility. The externalized DNA (%) refers to the amount detected at 85°C. (C, D) Effect of inactivation on B19V DNA release (dissociation from the capsid). Viral DNA (%) in relation to the input is shown.

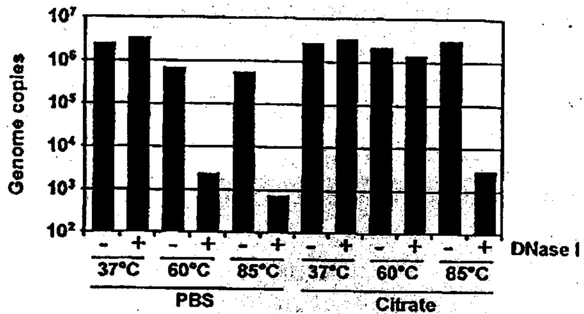

Fig. 3. Sensitivity of B19V DNA to DNase I after heat treatment in PBS or in citrate buffer.

voviruses. Although 60°C treatment leads to the externalization of nearly all the B19V genomes, the externalization in the rest of the tested viruses was at approximately 20 percent and in the range of 40 to 80 percent at 70°C. These results imply that the reason for the faster inactivation of B19V is due to the higher instability of its DNA in the encapsidated state.

DISCUSSION

To date, the lack of an appropriate cell culture to propagate B19V has complicated the experimental work with this virus. In contrast, optimal cell systems are available for many animal parvoviruses. For this reason, they are commonly used in validation studies as models for B19V. For an unknown reason, however, B19V has been shown to be more easily inactivated than the other members of the *Parvoviridae* family.[22,23] Therefore, the animal parvoviruses do not mimic the effect of inactivation procedures on B19V.[32] Although different inactivation conditions for B19V have been described, the underlying mechanism of the inactivation and the reason for its higher vulnerability to physicochemical conditions have not yet been elucidated.

In this study we have examined the structural capsid rearrangements occurring during the inactivation of B19V. For this purpose, we have applied two different procedures previously shown to efficiently inactivate B19V.[18,21,27] One is the exposure of the virus to heat (60°C for 10 min) and the other is the exposure to acidic conditions (pH 4 for 2 hr). Our results demonstrated that the first structural transition determining B19V inactivation is not the disintegration of the capsid, which remained intact (Fig. 1), but the loss of the viral DNA (Fig. 2). Interestingly, the heat sensitivity of B19V largely depends on the composition of the buffer. In a recent report, it was shown that a solution containing citrate conferred heat resistance to B19V.[29]

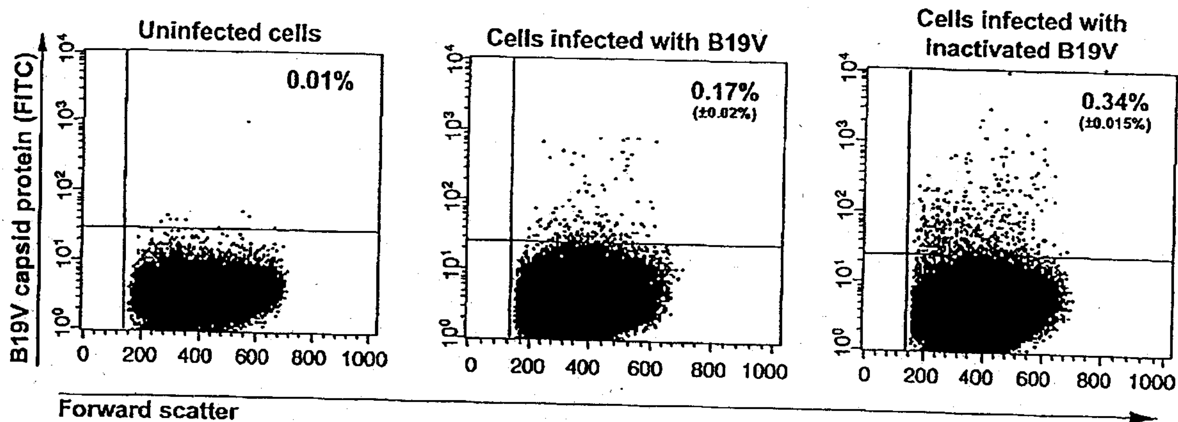

Fig. 4. Capacity of inactivated virus to bind to susceptible cells. UT7 cells were infected with either untreated or heat-inactivated B19V. The proportion of cells with bound virus was determined with FACS and is shown in the upper right quadrant of each panel. The percentages represent the mean ± SD of three separate experiments.

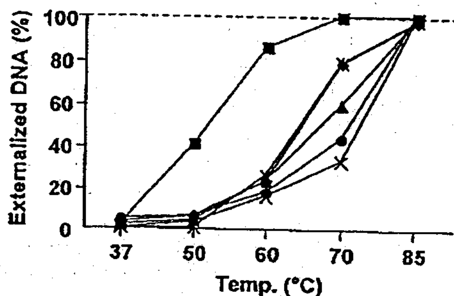

Fig. 5. DNA externalization pattern of different parvoviruses in response to increasing temperatures for 3 minutes. The amount of externalized DNA was quantified with the hybridization-extension assay. Values of DNA (%) refer to the total amount detected after 85°C treatment, which was in the range of 10^5 to 10^6 molecules per microliter for all viruses. (■) B19V; (●) bovine parvovirus; (◆) canine parvovirus; (▲) H1 parvovirus; (×) MVM; (✕) porcine parvovirus.

In our studies, we have confirmed this observation and found that in the presence of citrate, the viral DNA remains encapsidated (Fig. 3).

The release of the viral DNA in response to heat treatment was also detected in other parvoviruses (Fig. 5). Quantitative studies revealed that the kinetics of DNA externalization were surprisingly similar in all tested viruses with the exception of B19V, where it occurred prematurely (Fig. 5). The remarkable instability of the viral DNA in its encapsidated conformation explains the lower resistance of B19V to inactivation treatments.

The mechanism by which the intracellular environment destabilizes the parvovirus particles resulting in the release of the viral DNA is not fully understood. Growing evidence, however, indicates that parvovirus uncoating is performed without the need to disassemble the highly rigid capsid.[28,33-35] A series of capsid transitions triggered by the low endosomal pH seems to play a critical role by rendering the capsid flexible enough to allow the release of the viral DNA.[35] Among these transitions is the exposure of N-VP1. Increasing experimental evidence suggests that the conformational change leading to N-VP1 externalization leads also to DNA externalization.[33,34] Sustaining this notion is the observation that under mild acidification (pH 5), B19V externalizes N-VP1 sequences, and the viral DNA becomes accessible although mostly associated with the capsid.[28,36] In contrast, low pH treatment of MVM externalizes neither the N-VP1[34] nor the viral DNA.[28] Figure 6 represents schematically the progressive capsid rearrangement steps occurring during the inactivation of B19V.

As a result of the inactivation conditions applied in the present study, two major viral components were generated, empty capsids and free viral DNA, which might still have certain biologic activity. It has been recently shown that free genomic Kilham rat virus DNA induces innate immune activation and autoimmune diabetes through the TLR9 pathway;[37] however, whether B19V DNA or capsid proteins stimulate the innate immune system is not known. It has been increasingly acknowledged that pathogenic manifestations of B19V can also be elicited by the virus capsid proteins alone without infection. For instance, it has been shown that VP2 proteins are able to block hematopoiesis in vitro and in vivo.[38] The phospholipase A2 (PLA_2) activity of B19V is thought to contribute to inflammatory and autoimmune manifestations[39,40] and is suspected to be responsible for the arthropathies caused by B19V as well.[41] Although internal in native capsids, the VP1-PLA_2 motif becomes accessible upon exposure to

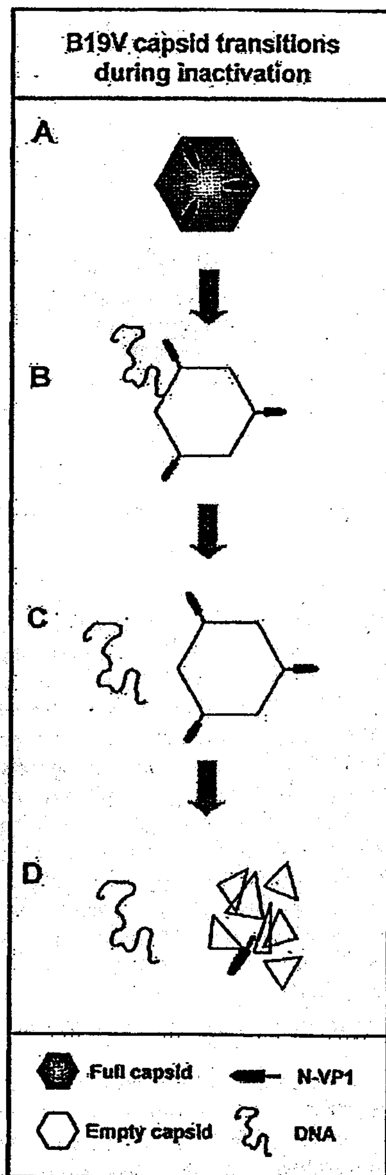

Fig. 6. Schematic representation of the B19V capsid structural transitions during inactivation. The first structural rearrangements observed after mild heat or low-pH treatments of B19V is the externalization of N-VP1 sequences,[36] including the PLA_2 motif and the accessibility of the viral DNA.[28] At higher temperatures or more acidic conditions, the viral DNA is dissociated from the capsid. Finally, the viral particle is disintegrated.

heat or low pH.[28] Therefore, although not infectious, the inactivated capsids are enzymatically active. The binding of the PLA_2-active capsids to cells (Fig. 4), whether specific or not, might still have certain biologic effect. It seems very unlikely, however, that such effects could be elicited through the administration of plasma-derived products containing inactivated B19V intact capsids. First, there may not be any intact capsids present in plasma-derived products due to the application of procedures of virus removal and/or inactivation, which are by far stronger than the ones applied in the present studies. Second, to elicit biologic activities other than virus replication, a large amount of B19V capsids or genomic viral DNA would be required. Synoviocyte migration for instance has been shown only to occur at a concentration of 10^{11} virions per mL.[41] Also, Norbeck and colleagues[38] use 10^{12} protein molecules per mL in an assay that showed the inhibition of hematopoiesis by VP2. Such high concentrations are simply not possible in plasma-derived products.

In summary, the molecular mechanism underlying the inactivation of B19V has been elucidated. The first structural transition determining B19V inactivation is not the disintegration of the capsid but the release of the viral DNA. Comparison studies revealed that although the DNA release from intact capsids seems to be a common feature within the *Parvoviridae* family, it occurs much more promptly and to a higher extent in B19V, explaining its lower resistance to inactivation treatments.

ACKNOWLEDGMENT

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識別番号・報告回数			報告日	第一報入手日 2007 年 11 月 29 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	Vox Sanguinis 2007; 93: 341-347	公表国 イギリス
販売名 (企業名)	ハプトグロビン注ーヨシトミ(ベネシス)			

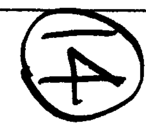
研究報告の概要

<背景及び目的>パルボウイルスは小さな非エンベロープの DNA ウイルスで、ウイルス不活化処理に対して比較的抵抗性がある。最近確認されたヒトパルボウイルス PARV4 が、類縁のジェノタイプ 2 型ウイルス（PARV5）を含め、血漿分画製剤の製造に使用されたプール血漿に混入していることが分かった。本報告では PARV4 が凝固因子製剤中に存在するのか否かを決定するための調査について述べる。
<材料及び方法>過去 30～35 年間に製造された第Ⅷ因子製剤について PARV4 及び B19 シーケンスのスクリーニングを実施した。PARV4 陽性製剤中の PARV4 ウイルス量は TaqMAN 分析法で測定し、DNA シーケンス分析によりジェノタイプを確認した。
<結果>第Ⅷ因子製剤 175 ロットのうち 28 ロットが PARV4 シークエンスを含み、その内 2 ロットにジェノタイプ 1 型及び 2 型の両方が存在することが分かった。最大ウイルス量は 10＾5copies/mL 以上であった。PARV4 陽性の第Ⅷ因子製剤の大部分は 1970 年代及び 1980 年代に製造されていた。B19 もまたこれらの製剤をしばしば汚染していた。
調査した 175 ロットの PARV4 DNA 陽性ロットを有効期間別に区分すると、有効期間が 1974-1989 年のロットでは 23%（27/115）が陽性であったのに対して、1990-2005 年では 2%（1/60）が陽性であった。
<結論>PARV4 は第Ⅷ因子製剤の 16%、特に 1970 年代及び 1980 年代の古いロットから検出された。これらの製剤からのウイルス安全性及びレシピエントへの感染可能性の重要性は、依然不明である。

使用上の注意記載状況・その他参考事項等

1. 慎重投与（次の患者には慎重に投与すること）
(4) 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。〕
(5) 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。
5. 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。〕

報告企業の意見	今後の対応
過去 30～35 年間に製造された第Ⅷ因子製剤から PARV4 シークエンスが検出されたとの報告である。 PARV4 が発見されたのは 2005 年であり、PARV4 及びその関連変異型である PARV5 の病原性は現時点では明らかではない。今後も注意深く PRV4 に関する追加情報をフォローする必要があると考える。	PRV4 に関連する情報については、今後も注視することとする。



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Human parvovirus PARV4 in clotting factor VIII concentrates

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Background and Objectives Parvoviruses are small non-enveloped DNA viruses, relatively resistant to virus inactivation procedures. The recently identified human parvovirus PARV4, including a related genotype 2 virus (also termed PARV5), has been found to be a contaminant of pooled plasma used in the manufacture of plasma-derived products. This report describes an investigation to determine whether PARV4 is present in clotting factor concentrates.

Materials and Methods Factor VIII concentrates manufactured in the past 30–35 years were screened for PARV4 and human parvovirus B19 (B19V) sequences. Viral loads in products testing positive for PARV4 were quantified using a consensus TaqMan assay designed to a highly conserved region. DNA sequence analysis was performed to confirm the genotypes present.

Results From a total of 175 lots of factor VIII concentrate, 28 of these contained PARV4 sequences, and in two lots both genotypes 1 and 2 were found to be present. The highest viral loads observed exceeded 10^5 copies per ml. The majority of factor VIII concentrates testing positive for PARV4 were manufactured in the 1970s and 1980s. Human B19V was also a frequent contaminant of these products.

Conclusions PARV4 was detected in 16% of factor VIII concentrates, particularly in older batches from the 1970s and 1980s. The significance in terms of the viral safety and potential transmission to recipients of these products is not yet known.

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Key words: factor VIII, genotype, PARV4, PARV5, parvovirus, virus contamination.

Introduction

PARV4 was originally identified in plasma from a patient with symptoms of acute virus infection following high-risk behaviour for human immunodeficiency virus 1 (HIV-1) transmission, but subsequently confirmed to be HIV-1 negative [1]. This patient was an intravenous drug user, infected with hepatitis B virus (HBV), with a range of symptoms including fatigue, vomiting and diarrhoea, sore throat, neck stiffness and joint pains. Phylogenetic analysis showed that PARV4 did not closely resemble other known human or animal parvoviruses [1].

Parvovirus B19 (B19V) is the prototype human parvovirus, infecting erythroid progenitor cells leading to erythema infectiosum, aplastic crisis, arthropathy and hydrops fetalis [2]. B19V is normally transmitted via the respiratory route; however, transmission also occurs through the administration of contaminated blood products and solvent/detergent-treated plasma and can result in clinically apparent infection [3–6]. Since 2004, European regulations have required that manufacturers of certain plasma derivatives, including anti-D immunoglobulin and plasma pooled and treated for virus inactivation, screen pooled plasma for B19V by nucleic acid amplification techniques (NAT), and this has led to a reduction in the levels of B19V present in manufacturing start pools [7]. NAT screening for B19V has now been widely implemented by manufacturers.

We have recently demonstrated the presence of PARV4 and a related variant virus (termed PARV5), in pooled plasma used in the manufacture of plasma-derived medicinal products [8]. These viruses are frequently detected in 4–5% of these pools with viral loads of up to 10^6 copies per ml of plasma.

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In the case of blood donors, PARV4 and PARV5 have been found in approximately 2% of individuals and at a higher frequency in febrile patients [9]. Sequence analysis shows that PARV4 and PARV5 share ~92% nucleotide identity over a 4860-bp region [10], similar to the level observed between B19V genotypes 1–3 [11], to which PARV4 shares ~45% nucleotide identity. At the amino acid level, PARV4 and PARV5 sequences are more conserved, and this is especially the case for the second open reading frame (ORF2), encoding the viral capsid-like protein, such that PARV4 and PARV5 are likely to represent a single serotype [10]. This sequence analysis has led to the proposal that PARV4 and PARV5 should be referred to by a single virus name, PARV4, comprising genotypes 1 and 2 (previously PARV5). In this study, we have investigated the presence of PARV4 genotypes 1 and 2 in clotting factor VIII concentrates, manufactured over the past 30–35 years. We have also examined these products for the presence of B19V.

Materials and methods

Factor VIII concentrates

Coagulation factor VIII concentrate products received at the National Institute for Biological Standards and Control (NIBSC) were stored at 4 to –20 °C until analysis. A total of 175 lots of 12 factor VIII concentrate products, from 10 manufacturers (named A–J), were investigated. Products were manufactured over a 30- to 35-year period, with expiry dates ranging between 1974 and 2005. Factor VIII product details are further described in Table 1.

Nucleic acid extraction

Factor VIII concentrates were reconstituted in sterile distilled water according to the manufacturer's instructions. Total nucleic acid was extracted from 1 ml of reconstituted concentrate using the MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany) and was eluted in 50 μl as previously described [7].

Screening for PARV4 in factor VIII concentrates

Factor VIII concentrates were initially screened for the presence of PARV4 genotype 1 and 2 sequences using a gel-based polymerase chain reaction (PCR), using primers specific to ORF2 of PARV4 [9]. We have previously confirmed the specificity and sensitivity of these primers to be one to 10 copies of PARV4 sequences. The presence of PARV4 in factor VIII concentrates was confirmed by DNA sequence analysis of amplification products. Amplicons were purified using the QIAEX Gel Extraction kit (Qiagen, Hilden, Germany). Sequencing was performed using the BigDye Terminator v1·1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), using the T7 promoter primer and the pUC/M13 reverse primer. Following removal of dye terminators, using the DyeEx 2·0 Spin Kit (Qiagen), sequencing reactions were run on an ABI 3130XL Genetic Analyser (Applied Biosystems).

Quantification of PARV4 in factor VIII concentrates

Following the initial screening of factor VIII concentrates for PARV4, viral loads in samples testing positive for these

Table 1 Detection of PARV4 and B19V in factor VIII concentrates

Product/ manufacturer	Expiry date	Number of lots tested	Purification process	Virus inactivation	Number of positive lots by PCR	
					PARV4	B19V
1/A	1974–1978	37	Precipitation	None	3	23
2/B	1976–1977	2	Precipitation	None	1	2
3/C	1976–1978	5	Precipitation	None	3	5
4/D	1977–1978	2	Precipitation	None	1	2
5/E	1977–1980	55	Precipitation	None	14	9
6/C	1985	1	Precipitation	Dry heat (68 °C, 72 h)	1	1
4/F	1985	1	Precipitation and adsorption	Wet heat (heptane) (60 °C, 20 h)	1	1
7/E	1985–1987	8	Precipitation and adsorption	Dry heat (68 °C, 72 h)	0	5
8/A	1986	4	Precipitation (plus further purification)	Steam treatment (60 °C, 10 h)	3	4
9/EGH	1997–2004	16	Monoclonal antibody	Pasteurization (60 °C, 10 h)	0	2
10/I	1998–2002	13	Monoclonal antibody	Solvent/detergent	0	7
11/I	1999–2003	13	Precipitation	Dry heat (80 °C, 72 h)	1	7
12/J	2001–2005	18	Affinity chromatography	Solvent/detergent, dry heat (80 °C, 72 h)	0	2
				Total number of positive lots/number of lots tested	28/175	70/175

130

viruses were determined using a real-time PCR assay designed to a highly conserved region of PARV4 as previously described [9,10]. The primers used in this assay are directed towards a region of ORF2 of PARV4 that is highly conserved between the two genotypes. A standard curve was generated from plasmid DNA containing the 103-bp ORF2 PCR product.

Detection of B19V DNA in factor VIII concentrates

Coagulation factor concentrates were additionally tested for the levels of B19V DNA using an in-house PCR assay as previously described [7]. This assay detects B19V genotypes 1–3.

DNA sequence analysis of a variable region of ORF1 of PARV4

Using a multiple sequence alignment of near full-length PARV4 genomes (GenBank accession no. DQ873386-91) [10], primers were designed to a variable region of the PARV4 genome. Primers PARV35F (5′ TTCCTACTGGATTTCTCTCCAACC 3′) and PARV596R (5′ GGTAAGGCAATAGCACCTTGAGG 3′) were used to amplify a 562-bp region of ORF1 of PARV4 (corresponding to nucleotides 317–878 of PARV4 genotype 1, GenBank accession no. AY622943, and nucleotides 151–712 of PARV4 genotype 2, GenBank accession no. DQ873390), from extracted factor VIII samples. Amplification reactions were performed using the proof-reading enzyme Phusion™ Hot Start DNA Polymerase (Finnzymes OY, Espoo, Finland) as described previously [8]. For thermal cycling, a T3 thermal cycler (Biometra, Göttingen, Germany) was used with the following cycling conditions: 98 °C for 30 seconds, followed by 45 cycles of 98 °C for 10 seconds, 59 °C for 30 seconds and 72 °C for 20 seconds. Amplicons were analysed by agarose gel electrophoresis and compared with known size markers. Amplification products were purified as before, and cloned into the pT7 Blue vector according to the manufacturer's instructions (Novagen, Darmstadt, Germany). Sequencing was performed as previously described and was analysed using the GCG software package, version 10·2 (University of Wisconsin, Madison, WI, USA). Sequences were aligned using Clustal W [12], and a neighbour-joining tree (nucleotide distance with Jukes–Cantor correction, pairwise gap deletion) with bootstrap resampling (100 replicates), was constructed using MEGA3 software [13].

Results

Contamination of factor VIII concentrates with human parvoviruses

A total of 175 lots of 12 factor VIII concentrate products, from 10 manufacturers, were examined for the presence of PARV4 and B19V DNA by PCR. The expiry dates on these lots

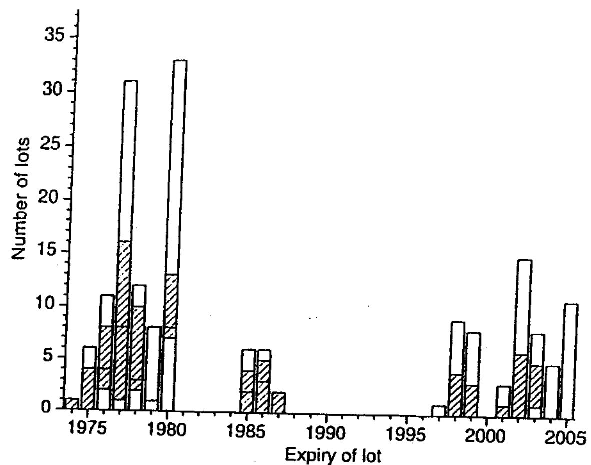

Fig. 1 Prevalence of parvoviruses PARV4 and B19V in factor VIII concentrates manufactured over the past 30–35 years. The number of lots testing positive for PARV4 (▭), B19V (▨), both PARV4 and B19V (▨), and those testing negative for these viruses (▭) are shown.

ranged from 1974 to 2005. As shown in Table 1, 16% (28/175) of lots tested positive for PARV4, while 40% (70/175) of lots tested positive for B19V DNA. The majority of factor VIII products testing positive for PARV4 DNA had an expiry date of pre-1990 [23% (27/115) of lots expiring 1974–1989 tested positive for PARV4, while only 2% (1/60) of lots expiring 1990–2005 tested positive for PARV4 DNA] (Fig. 1). In contrast, there was no significant difference in the prevalence of B19V in factor VIII products expiring pre- and post-1990 [45% (52/115) of lots expiring 1974–1989 tested positive for B19V, while 30% (18/60) of lots expiring 1990–2005 tested positive for B19V DNA] (Fig. 1).

PARV4 ORF2 PCR products amplified by the gel-based assay were sequenced, and the majority determined to be of PARV4 genotype 2 (Table 2). In two factor VIII products both PARV4 genotype 1 and 2 sequences were amplified and sequenced. Viral loads of PARV4 in factor VIII products were determined by a consensus sequence real-time PCR assay [9], designed to detect a highly conserved region of ORF2 of PARV4. Viral loads ranged from < 100 to more than 3×10^5 copies per ml of product (Table 2), with the majority of contaminated lots containing 4–5 $\log_{10}$ PARV4 copies per ml of product (Fig. 2). The levels of B19V were as high as $2{\cdot}5 \times 10^8$ IU/ml of product (Table 2).

Manufacturing plasma pools relating to these factor VIII products were only available for the most recent factor VIII products. Factor VIII product number 28 (Table 2) had an expiry date of 2003, and was manufactured from two plasma pools 28A and 28B. Plasma pool 28A tested positive for PARV4 genotype 1 DNA by PCR with a viral load of $3{\cdot}3 \times 10^5$ copies per ml of plasma, while pool 28B tested negative for both PARV4 genotypes.

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Table 2 Levels of PARV4 and B19V in factor VIII concentrates testing positive for PARV4 DNA

Factor VIII	Product/ manufacturer	Expiry date	PARV4 genotype[a]	PARV4 viral load ($\log_{10}$ genome copies per ml product)	B19V viral load ($\log_{10}$ IU/ml product)
1	1/A	1976	2	< 2·00[c]	8·40
2		1977	1	1·89	6·71
3		1977	1 & 2[b]	1·71	7·64
4	2/B	1977	2	3·11	2·59
5	3/C	1976	2	1·82	4·91
6		1977	2	3·28	5·33
7		1978	1	1·86	2·75
8	4/D	1977	2	2·48	2·22
9	5/E	1977	2	1·75	–
10		1977	2	4·10	2·39
11		1977	2	4·82	6·05
12		1978	2	4·15	–
13		1978	2	4·36	–
14		1979	2	2·66	–
15		1980	1	4·31	6·44
16		1980	1 & 2	3·01	–
17		1980	2	4·39	–
18		1980	2	5·49	–
19		1980	2	5·03	–
20		1980	2	2·37	–
21		1980	2	4·30	–
22		1980	2	2·00	–
23	4/F	1985	1	< 2·00[c]	4·57
24	6/C	1985	1	1·32	5·79
25	8/A	1986	1	4·08	7·15
26		1986	2	3·81	5·85
27		1986	2	4·53	4·36
28	11/I	2003	1	2·32	–

[a]Determined by sequencing of ORF2 amplification products.

[b]ORF2 amplification products were determined to be PARV4 genotype 1 sequences, while the amplified variable ORF1 region was determined to be PARV4 genotype 2.

[c]Factor VIII lot tested positive for PARV4 DNA by qualitative PCR but the viral load was below the level of quantification by real-time PCR, and was therefore given an arbitrary viral load of < 2 $\log_{10}$ genome copies per ml product.

–, product tested negative for B19V DNA.

Analysis of PARV4 sequences

Previous analysis of PARV4 sequences showed that ORF1 was slightly less conserved than ORF2 [10]. We therefore amplified and sequenced a 562-bp variable region at the 5′ end of ORF1 from 26/28 factor VIII concentrates testing positive for PARV4 sequences. It had not been possible to amplify the 562-bp variable ORF1 region of PARV4 from factor VIII products 7 and 9 (Table 2). Both PARV4 genotype 1 and 2 sequences were amplified from factor VIII product number 16 (Table 2). Phylogenetic analysis of these PARV4 sequences shows that they fall into two distinct genetic clusters, representing genotypes 1 and 2 (Fig. 3). Across the two genotypes, PARV4 nucleotide sequences amplified from factor VIII products differ from each other by greater than 11% over the region sequenced. Within each genotype, all PARV4 sequences amplified from factor VIII concentrates were greater than 99% homologous (at the nucleotide level, over the 515-bp region sequenced), despite products being manufactured over a 30- to 35-year period. In fact, several PARV4 genotype 1 and 2 sequences amplified from factor VIII products manufactured as early as the mid-1970s were 100% identical at the nucleotide level, over the 515-bp region sequenced, to the recently identified respective strains BR10749 (genotype 1) and BR10627 (genotype 2) [10].

Discussion

We recently demonstrated the presence of the newly identified human parvovirus PARV4 including the related genotype 2

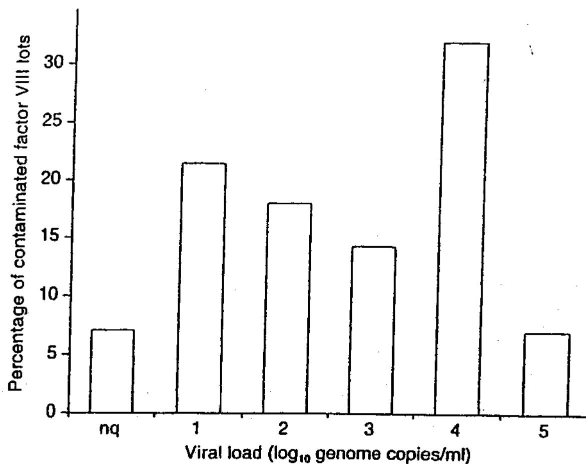

Fig. 2 Viral DNA loads of PARV4 ($\log_{10}$ genome copies per ml) in contaminated factor VIII concentrates. nq, not quantifiable.

virus (previously termed PARV5) in manufacturing plasma pools, with these viruses detected in approximately 5% of pools [8,9]. In this present study, we have detected PARV4 viruses in products derived from such plasma pools, specifically in coagulation factor VIII products, manufactured over the past 30-35 years. Information regarding the source of plasma used in the manufacture of products examined in this study was difficult to obtain as it is not provided with the products. These details could only be obtained for the most recent factor VIII product testing positive for PARV4 DNA. This factor VIII concentrate had an expiry date of October 2003, and was manufactured from two plasma pools in September 2000. Donations relating to these plasma pools were collected in or after July 1998 from paid donors from the USA. This suggests that viruses detected in these factor VIII products may date from up to 5 years prior to the expiry date on the product. Details from other manufacturers of recent factor VIII concentrates (testing negative for PARV4) also indicate that donations relating to these products were sourced up to 5 years prior to the expiry date.

The prevalence of PARV4 in factor VIII concentrates was found to be greater in products expiring pre-1990 than in those with an expiry date of post-1990. This difference in the prevalence of PARV4 in factor VIII products over time may reflect the introduction of blood safety measures from the mid-1980s in response to the HIV epidemic, in particular, the introduction of screening tests for HIV and hepatitis C virus (HCV) (in 1986 and 1991, respectively), and virus inactivation of manufacturing plasma pools (introduced in the mid-1980s). The screening of blood donations for HIV and HCV identified 'high-risk' donor groups, such as homosexual males and individuals with a history of intravenous drug use (IVDU), and these groups were subsequently excluded from donating blood [14]. Factor VIII products tested in this study

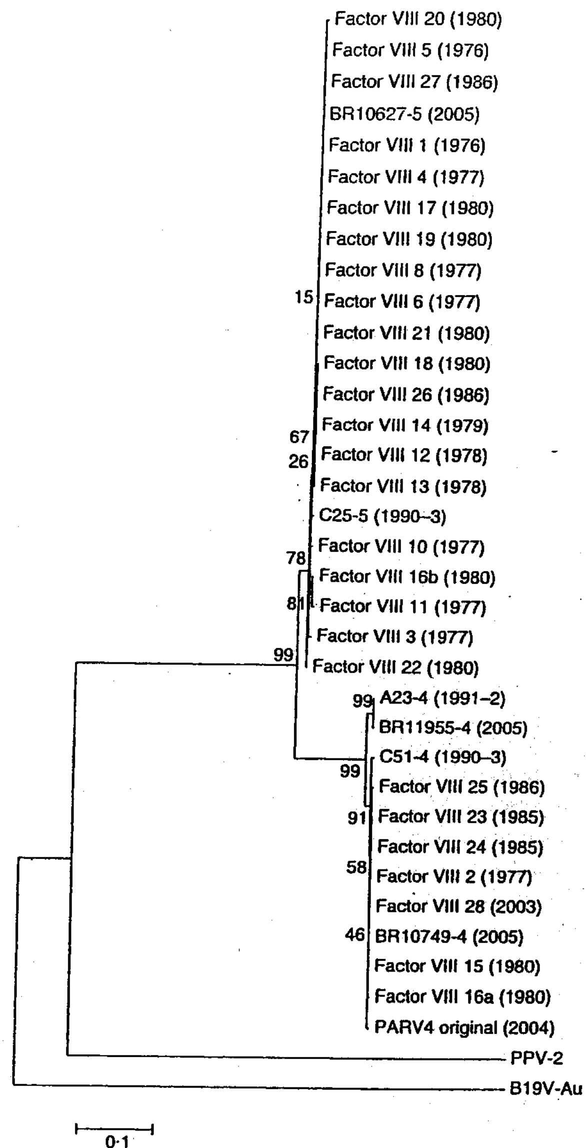

Fig. 3 Phylogenetic analysis of a 515-bp region of ORF1 of PARV4 amplified from factor VIII concentrates. Sequences are named according to factor VIII number and expiry of lot (Table 2). The alignment includes other recently sequenced strains of PARV4 genotype 1; PARV4 original (GenBank accession no. AY622943), BR10749-4 (GenBank accession no. DQ873386), BR11955-4 (GenBank accession no. DQ873388), A23-4 (GenBank accession no. DQ873389) and C51-4 (GenBank accession no. DQ873387); and PARV4 genotype 2, BR10627-5 (GenBank accession no. DQ873390) and C25-5 (GenBank accession no. DQ873391). The PARV4 original strain was sourced from the index case patient in 2004 [1]. Strains BR10749-4 and BR10627-5 were identified in our preliminary study of plasma pools [8], while the other strains were identified in further screening studies of manufacturing plasma pools [9]. Strains BR10749-4, BR11955-4 and BR10627-5 were from plasma samples received at NIBSC between 2004 and 2005, while A23-4, C51-4 and C25-5 were received at NIBSC 1990–1993. The alignment also includes the corresponding nucleotide sequences of porcine parvovirus 2 (PPV-2) (GenBank accession no. AB076669) and B19V-Au genotype 1 virus (GenBank accession no. M13178) as outgroups. Genetic distance and bootstrap values are indicated.

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with an expiry date of post-1990 are likely to originate from plasma sourced from screened 'low-risk' blood donors. Therefore, the reduced prevalence of PARV4 in more recently manufactured factor VIII products may be a result of the removal of specific 'high-risk' donor populations.

Virus inactivation using a variety of heat treatments was introduced into the manufacturing process of existing coagulation factor products in the mid-1980s, before the implementation of HIV and HCV screening. The effectiveness of these treatments, for HCV particularly, varied greatly, depending on the duration and temperature of heating and whether the product is in liquid form or lyophilized [15,16]. Other virus inactivation procedures include solvent/detergent treatment, which is effective against enveloped viruses [17,18]. Animal parvoviruses, such as canine, bovine and porcine parvoviruses, and minute virus of mice, were used to investigate the effectiveness of virus inactivation of plasma prior to the development of cell culture-based assays for B19V. By virtue of their small size and absence of viral envelope, animal parvoviruses are relatively resistant to inactivation by a range of heat and chemical agents [19]. Based on studies using these model parvoviruses, B19V was also expected to be resistant to these virus inactivation strategies and unlikely to be effectively eliminated by dry heat and pasteurization [5]. However, recent studies using B19V cultures suggest that it is more susceptible to heat and low pH treatments than other animal parvoviruses [20–23]. Results here show that there was not a significant reduction in the prevalence of B19V DNA in factor VIII products manufactured after the introduction of virus inactivation procedures (B19V DNA was detected in 41% of products manufactured without virus inactivation measures vs. 39% of products manufactured using virus inactivation steps). However, it must be noted that virus inactivation procedures such as heat and low pH treatments do not physically remove viral DNA, which may still be detectable by NAT. The effect of virus inactivation procedures on PARV4 remains to be determined; however, the reduced prevalence of PARV4 in factor VIII products manufactured with virus inactivation (8% in virus inactivated products vs. 22% in products manufactured without virus inactivation) may suggest that these viruses are susceptible to virus inactivation treatments. The increased prevalence of PARV4 in factor VIII concentrates expiring in the late 1970s and mid-1980s may also result from epidemics of infection as has been observed for B19V [2]. Our investigation of recent and archived manufacturing plasma pools for PARV4 identified an increased prevalence of these viruses in plasma pools received from one manufacturer between 1991 and 1992, which may be the result of seasonal and/or epidemic variation [9].

PARV4 viral loads in these factor VIII concentrates were as high as 5 $\log_{10}$ per ml of product, while the levels of B19V were as high as 8 $\log_{10}$ per ml of product. The higher levels of contaminating PARV4 and B19V viruses were confined to the older factor VIII concentrates (expiring pre-1990). Considering that downstream purification and processing of manufacturing plasma pools will alter the viral loads present in subsequent plasma-derived products, viral loads in these factor VIII concentrates correlate well, albeit being approximately 1 $\log_{10}$ lower, with the levels of PARV4 and B19V detected in recent and archived plasma pools [8,9]. In these manufacturing plasma pools, the viral loads of these viruses typically range up to 6 $\log_{10}$ per ml of plasma for PARV4, and up to 9 $\log_{10}$ per ml of plasma for B19V.

In manufacturing plasma pool samples previously examined for the presence of PARV4, we found that genotypes 1 and 2 were detected in approximately equal proportions [8,9]. These samples were received at NIBSC for plasma pool testing between 2005 and 2006, but also included archived samples received between 1990 and 1993. In this present study, we detected a greater prevalence of PARV4 genotype 2 over genotype 1 in factor VIII concentrates manufactured in the past 30–35 years (21 products testing positive for PARV4 genotype 2 sequences vs. nine products testing positive for PARV4 genotype 1 sequences). As the majority of these PARV4-positive factor VIII products had expiry dates of pre-1990 and were likely to have been manufactured from blood donations collected before the mid-1980s, these results suggest a temporal change in the prevalence of PARV4 genotypes over the past 30–35 years. A similar temporal change in parvovirus genoprevalence has been suggested in the case of B19V genotypes 1 and 2, where both genotypes were equally detected in the tissues of individuals born in the 1950s or earlier, while genotype 1 viruses were predominantly detected in the tissues of individuals born in the 1960s and later [24]. Further evidence for a temporal succession of infection with PARV4 genotype 1 over genotype 2 has recently been reported in HIV infected patients [25].

Although positive PCR results do not necessarily reflect infectivity, the detection of PARV4 DNA in coagulation factor VIII concentrates in this study raises questions as to whether PARV4 has been transmitted parenterally to the recipients of such products. PARV4 was originally identified in an individual who was a daily injecting drug user and it is possible that he acquired the virus through this route [1]. In addition, we have identified an increased incidence in the detection of PARV4 in febrile patients, including IVDUs and homosexual men [9], and in individuals infected with HCV (including IVDUs) [26]. An increased prevalence of PARV4 in HIV-infected individuals has also recently been reported [25]. Nothing is yet known as to whether there is any pathology associated with PARV4 infection. Although the PARV4 index case patient had an acute viral infection syndrome, the lifestyle of this individual and an underlying infection with HBV make it impossible to determine whether PARV4 played a role in his symptoms [1]. The presence of PARV4 in pooled plasma from healthy blood donors [illegible]

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may cause subclinical infections, and the implications for the safety of blood and plasma-derived products such as factor VIII are still not known.

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