資料２－２

Ａ　研究報告（詳細版）

平成２５年３月２９日
（平成２４年１１月～平成２５年１月受理分）

医薬品
医薬部外品　　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2012 年 9 月 19 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①② 抗 HBs 人免疫グロブリン		研究報告の公表状況	Vox Sanguinis 2012; 103(3): 183-185	公表国 中国	
販売名（企業名）	① 抗 HBs 人免疫グロブリン筋注 200 単位/1mL「日赤」（日本血液製剤機構） ② 抗 HBs 人免疫グロブリン筋注 1000 単位/5mL「日赤」（日本血液製剤機構）					

研究報告の概要

はじめに

パルボウイルスは、1 本鎖の DNA をもつ小さいノンエンベロープウイルスである。これまで、少なくとも 4 つのパルボウイルス（アデノ随伴ウイルス（AAV）、ヒトパルボウイルス B19（B19V）、パルボウイルス 4（PARV4）、ヒトボカウイルス（HBoV））がヒトを感染させることが知られている。これら 4 つのウイルスの中で、B19V は血液及び血液製剤のウイルス安全性に対して明らかなリスクが知られている。2005 年に、PARV4 そのものの感染症なのか、共感染症によるものなのかは明らかになっていないが、PARV4 が急性ウイルス感染症の多様な症状を発症した患者の血漿検体から初めて確認された。これまでに 3 つの PARV4 遺伝子型は確認されている。B19V と同様に、PARV4 も製造用血液製剤に使用される血漿プールの夾雑物（コンタミ）として確認された。ヨーロッパと北米からの製造用血漿プールの広範囲な調査では、PAVA4 は近年の原料プールの 4%（14/351）で検出された。しかしながら、中国の原料血漿プールの PAVA4 夾雑物の程度と量については確認されていない。本研究では、我々は中国で集められた血漿からつくられる原料血漿プールにおける PAVA4 の感染状況を調査した。

方　法

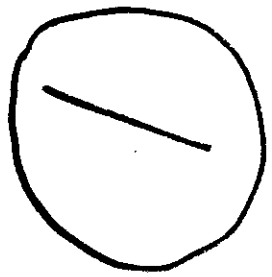
2007 年から 2010 年の間に、3 つの製造業者で集められた合計 195 の原料血漿プールを PARV4 の検査をした。原料プールに混ぜられるそれぞれの血漿は、プールの前に ELISA により抗 HIV 抗体、HBs 抗原と抗 HCV 抗体の検査をした。全ての血漿は HIV、HBV 及び HCV に感染していないことが確認された。製造業者 A、B と C は、それぞれ中国中央部、中国北部、中国南西部に位置する。それぞれのサンプルに対して、High Pure Viral Nucleic Acid Kit（ロシュ、ドイツ）を用いて、血漿 200μL から 50μL の核酸を抽出した。前述したとおり、5'-CTAAGGAAACTGTTGGTGATATTGCT-3' と 5'-GGCTCTCCTGCGGAATAAGC-3' のプライマーと特定の保存性の高い PARV4 の ORF2 領域（nucleotides 3258-3387, GeneBank, accession no.AY622943.1）にある 5'-(FAM)TGTTCAACTTTCT CAGGTCCTACCGCCC(TAMRA)-3' のプローブを用いてリアルタイム定量 PCR を行った。それぞれの PCR には、Premix Ex Taq™(1X)(TaKaRa, Dalian, China), それぞれのプライマー0.75nM、プローブ 0.5nM と DNA テンプレート 3μL を最終液量 20μL として用いた。PCR には、CFX96Real-Time PCR Detection System(Bio-Rad, CA, USA)用いて実施した。その時のサーマルサイクル条件は、プレインキュベート 95℃10 分の後、42 サイクル（95℃30 秒、52℃30 秒、68℃30 秒）で行い、最後に 68℃10 分とした。結果は、B_{IO}RAD CFX Manager software を用いて解析を行った。

標準曲線は、103-bp の PCR 産物を含むプラスミドの階段希釈により得た。検出限界は反応ごとに 10 copies であった。プライマーは特異性が高く、核酸増幅検査のための（code09/110）パルボウイルス B19 ジェノタイプに対する第 1 回 WHO 国際リファレンスパネルの抽出された核酸に反応しなかった。陽性のサンプルは、pMD18-T ベクターでクローニングをおこない、ABI3730 とアクセサリーを用いてシーケンスを行った。その後、シーケンス結果を DNASTAR ソフトウエアパッケージを用いて単離された PARV4 プロトタイプ（GenBank accession no. AY622943.1）との同一性を確認した。

使用上の注意記載状況・その他参考事項等

抗 HBs 人免疫グロブリン筋注 200 単位/1mL「日赤」
抗 HBs 人免疫グロブリン筋注 1000 単位/5mL「日赤」

血液を原料とすることに由来する感染症伝播等
vCJD 等の伝播のリスク



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結　果

結果は、検査したロットの 26.15% (51/195) が PARV4 陽性を示した。3 つの製造業者からの原料血漿プールにおける PARV4 DNA の陽性率は、有意差があった。製造業者 A からのロットの 38.61%は PAVA4 陽性、一方、製造業者 B と C の陽性率は、それぞれ 15.00%と 12.16%であった。陽性検体の増幅産物のシーケンスと照合を行った結果から 100%、若しくは 1 塩基違いのほぼ 100%の同一性を明らかにした。

標準曲線の勾配をもとに、増幅効率は 95.2%であった。血漿プール中の PARV4 DNA 量を測定した。結果は、DNA の量は血漿中に 2.83×10^{3}copies/mL から 2.35×10^{7}copies/mL の範囲であることを示した。3 つの製造業者からの血漿プールのそれぞれのバッチは、2000 から 8300 の個別血漿からなる。結果は、もしウイルス DNA の高い混入量を持つ一つの個別血漿が血漿プールに存在（コンタミ）した場合、ウイルス量は血漿中に $10^{10}-10^{11}$copies/mL になることが示唆された。

考　察

B19V と潜在的に PARV4 は、輸血の安全性に関わっている。B19V は様々な疾患の原因となり、頻繁に血漿プール中で高いウイルス量が検出されるために、血漿たん白質治療協会 (PPTA) と FDA は、製造に用いられるプール中の B19V の量を制限するためにミニプールスクリーニングを要求している。PARV4 に感染しているもともとの患者は、急性ウイルス感染症を起こしているかもしれないが、これまで PARV4 に関連した病気は確認されていない。血漿プール中の PARV4 の混入量を制限するための国際的、或いは国内機関からの指針は出されていない。血漿プール中の PARV4 の高い混入量は輸血安全性の潜在的なリスクになるかもしれない、そのため PARV4 の病原性に関する更なる研究が至急に求められている。

これまでの二つの報告では、血漿由来医薬品の製造に用いた血漿プール中の PAVA4 の存在を研究した。これらの 2 つの研究結果は、ほとんどの製造業者のプール血漿中の PARV4 DNA の陽性率は低く、3 つの製造者からの結果は例外としても、PARV4 DNA は、30%以上のプール血漿で陽性であった。本報告では、PARV4 DNA の検出頻度は高く、特に製造業者 A では、38.61%という高値であった。この結果は、季節的な及び/或いは地理的な流行の変化による結果なのかもしれない。

最近の報告では PARV 4 感染は、HIV、HCV 及び/或いは HBV 感染に関連していることが示されている。これらの結果は、汚染した血液及び/或いは静脈注射剤使用（静注剤）を介した PARV4 伝播を示唆している。我々の研究において、検査を行ったプール血漿は、HIV/HCV/HBV に感染していない個々の健康な個別血漿が数千単位でプールされたものであり、また、HIV/HCV/HBV の感染に関係していないプール血漿中の PARV4 DNA の存在を明らかにした。HIV/HCV/HBV 感染集団、或いは他のハイリスク集団における PARV4 の増加している有病率は、感染に対して暴露される可能性が高くなるかもしれない、しかし PARV4 の持続的な存在はこれらの集団に限定されたものではないことが、以前の報告で確認されている。PARV4 の生物学的性質と感染経路に対して更なる研究をしていく必要がある。

報告企業の意見	今後の対応
ヒトパルボウイルス 4 (PARV4) は、ヒトパルボウイルス B19 の近縁ウイルスで、ゲノムに DNA をもつノンエンベロープウイルスで、大きさは 20nm 程度と比較的小さい。その病原性は現時点で明らかではないが、血漿分画製剤からの伝播が報告されていることもあり、今後、注意深く追加情報をフォローする必要があると考えている。	PARV4 に関する追加情報の入手に努める。

VoxSanguinis
The International Journal of Transfusion Medicine

JBPO 2012-001
ISBT International Society of Blood Transfusion

Vox Sanguinis (2012) 103, 183–185

DOI: 10.1111/j.1423-0410.2012.01605.x

SHORT REPORT

Human parvovirus PARV4 in plasma pools of Chinese origin

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Vox Sanguinis

Received: 20 June 2011, revised 17 February 2012, accepted 21 February 2012, published online 27 March 2012

Human parvovirus 4 (PARV4) is present in blood and blood products. As the presence and levels of PARV4 in Chinese source plasma pools have never been determined, we implemented real-time quantitative PCR to investigate the presence of PARV4 in source plasma pools in China. Results showed that 26·15% (51/195) of lots tested positive for PARV4. The amounts of DNA ranged from $2{\cdot}83 \times 10^3$ copies/ml to $2{\cdot}35 \times 10^7$ copies/ml plasma. The high level of PARV4 in plasma pools may pose a potential risk to recipients. Further studies on the pathogenesis of PARV4 are urgently required.

Key words: human parvovirus 4, real-time quantitative PCR, source plasma pools.

Introduction

Parvoviruses are small, non-enveloped viruses, with a single-stranded DNA genome. So far, at least four parvoviruses are known to infect human beings, including adeno-associated virus (AAV), human parvovirus B19 (B19V), parvovirus 4 (PARV4) and human bocavirus (HBoV) [1]. Among these four parvoviruses, B19V is known to be a significant risk to the viral safety of blood and blood products [2, 3]. In 2005, PARV4 was originally identified in the plasma sample from a patient presenting with multiple symptoms of acute viral infections [4], although it was not known whether this was due to the viral infection itself or to be co-incidental. Three PARV4 genotypes have now been identified. Similar to B19V, PARV4 has also been demonstrated to be a contaminant of plasma pools used for manufacturing blood products [5]. In an extensive analysis of manufacturing plasma pools from Europe and North America, PARV4 was detected in 4% (14/351) of recently sourced pools [6]. However, the extent and level of PARV4 contamination of Chinese source plasma pools have not been determined. In this study, we investigated the infection status of PARV4 in source plasma pools from plasmapheresis donations collected in China.

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Supported by National Key Technology R&D Program (2008BAI54B08-4).

Methods

A total of 195 source plasma pools from three manufacturers collected between 2007 and 2010 were tested for PARV4. Each donation mixed in the plasma pools was tested for anti-HIV, HBsAg and anti-HCV by ELISA before pooling. All the donations were confirmed to be uninfected with HIV, HBV and HCV. Manufacturer A, B and C are located in central China, northern China and south-western China, respectively. For each sample, 50 μl of nucleic acid was extracted from 200 μl of plasma by using High Pure Viral Nucleic Acid Kit (Roche, Germany). As previously described, real-time quantitative PCR was performed with primers 5′- CTAAGGAAACTGTTGGTGATATTGCT -3′ and 5′-GGCTCTCCTGCGGAATAAGC-3′ and probe 5′-(FAM) TGTTCAACTTTCT CAGGTCCTACCGCCC (TAMRA) -3′ specific for a highly conserved region of ORF2 of PARV4 (nucleotides 3285–3387, GenBank accession no. AY622943.1) [6]. Each PCR contained Premix Ex Taq ™ (1×) (TaKaRa, Dalian, China), 0·75 nM each primer, 0·5 nM probe and 3 μl of template DNA in a final volume of 20 μl. PCRs were run on a CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA) with the following thermal cycle conditions: 10 min of preincubation at 95°C, 42 cycles in three steps each (95°C for 30 s, 52°C for 30 s, 68°C for 30 s) and one final cycle of 10 min extension at 68°C. Results were analysed with BioRAD CFX Manager software. Standard curves were generated with serial dilutions of a plasmid containing the 103-bp PCR product. The detection limit of this assay was found to be 10 copies per reaction. The primers were highly specific and non-reactive for nucleic acid extracted from

the 1st WHO International Reference Panel for Parvovirus B19 Genotypes for NAT-based assays (code 09/110). PCR products of the positive samples were cloned into pMD18-T vector and sequenced using ABI 3730 and accessories. Then the sequences were aligned with the sequence of PARV4 prototype isolate (GenBank accession no. AY622943.1) using DNASTAR software package.

Results

Results showed that 26·15% (51/195) of lots tested positive for PARV4. Positive rates of PARV4 DNA in source plasma pools from three manufacturers were significantly different ($P < 0·01$). 38·61% of lots from manufacturer A were positive for PARV4, while the positive rate of manufacturer B and manufacturer C was 15·00% and 12·16%, respectively (Table 1). Sequencing and alignment of amplification products of the positive samples revealed that they exhibited 100% identity or nearly 100% identity with 1 base difference (Fig. 1).

Based on the slope of the standard curve, we found that the amplification efficiency was 95·2%. We determined the PARV4 DNA loads in plasma pools. The result indicated that the amounts of DNA ranged from $2·83\times10^3$ copies/ml to $2·35\times10^7$ copies/ml plasma (Table 1). Each batch of the plasma pools from the three manufacturers consisted of 2000 to over 8300 donations. The result suggested that the virus load could be 10^{10}–10^{11} copies/ml plasma if one donation with high load of viral DNA was responsible for the contamination of the plasma pool.

Discussion

B19V, and potentially PARV4, are involved in transfusion safety. Since B19V causes various diseases, and is frequently detected at high levels in plasma pools, the Plasma Protein Therapeutics Association and FDA proposed to screen mini-pools and thus to restrict the quantity of B19V in the manufacturing pool [7, 8]. Although the 'original patient' who was infected with PARV4 did have acute viral infection syndrome, so far no diseases were confirmed to be related with PARV4. There is no standard issued by international or domestic organizations for restricting the level of PARV4 in plasma pools. The high level of PARV4 in plasma pools may cause potential risk to transfusion safety, so further study on the pathogenesis of PARV4 is urgently required.

In previous reports, two studies investigated the presence of PARV4 in plasma pools used in the manufacture of

Table 1 PARV4 DNA prevalence and levels in source plasma pools

	Number of lots (%)			
PARV4 DNA load (copies/ml)	Manufacturer A	Manufacturer B	Manufacturer C	Total
10^7–10^8	2 (1·98)	0	0	2(1·03)
10^6–10^7	8 (7·93)	1(5·00)	2(2·70)	11(5·64)
10^5–10^6	16 (15·84)	2(10·00)	0	18(9·23)
10^4–10^5	9 (8·91)	0	4(5·41)	13(6·67)
10^3–10^4	4 (3·96)	0	3(4·05)	7(3·59)
Positive-10^3	0	0	0	0
Number of positive lots	39 (38·61)	3 (15·00)	9 (12·16)	51 (26·15)
Number of negative lots	72(71·29)	17(85·00)	65(87·84)	154(78·97)
Number of lots tested	101	20	74	195

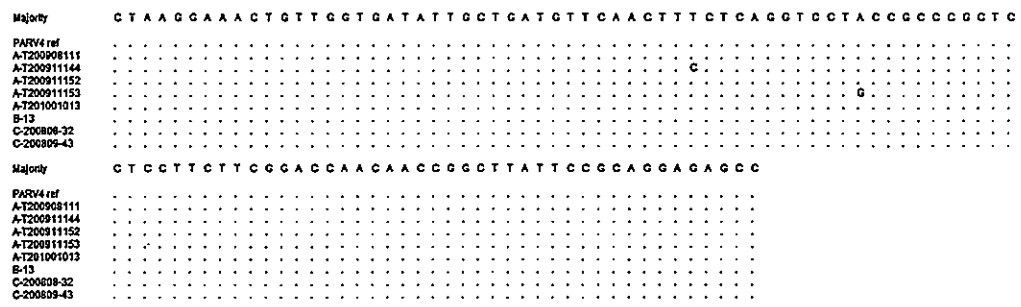

Fig. 1 Multiple sequence alignment of the PARV4 prototype isolate (GenBank accession no. AY622943.1), and isolates identified in pools, was created with the program CLUSTALW in DNASTAR software package.

plasma-derived medicinal products [5, 6]. The results in these two studies indicated that the positive rate of PARV4 DNA in plasma pools from most manufacturers was low, with the exception of those from three manufacturers, where more than 30% plasma pools tested positive for PARV4 DNA. In this report, we found that the frequency of detection of PARV4 DNA was high, particularly from manufacturer A which was as high as 38·61%. This may be the result of seasonal and/or geographical epidemic variation.

Recent studies have shown that PARV4 infection was in association with HIV, HCV and/or HBV infection [9–11]. These results implied PARV4 transmission via contaminated blood and/or intravenous drug use. In our study, the tested plasma pools contained thousands of plasma donations from healthy individuals uninfected with HIV/HCV/HBV, which demonstrated that the presence of PARV4 DNA in these plasma pools was not associated with the infection of HIV/HCV/HBV. The increased prevalence of PARV4 in HIV/HCV/HBV-infected groups or other high-risk groups may be due to increased exposure to infection, but the persistence of PARV4 was not exclusive to these groups, as has been observed in a previous report [12]. Further studies are needed to improve our knowledge of the route of transmission and biological properties of PARV4.

Acknowledgements

This work was supported by National Key Technology R & D Program (2008BAI54B08-4).

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化粧品

識別番号・報告回数			報告日	第一報入手日 2012 年 11 月 30 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	人ハプトグロビン		研究報告の公表状況	Chinese Journal of Biologicals 2012; 25(8): 1043-1044-1048	公表国 中国	
販売名 (企業名)	ハプトグロビン静注 2000 単位「ベネシス」 (日本血液製剤機構)					

研究報告の概要

ヒトパルボウイルス B19 は人に対し病原性を持つ既知の唯一のパルボウイルスである。個人の年齢および免疫状態の違いにより感染後の臨床像が異なり、小児の感染では伝染性紅斑、成人女性の感染では一過性の多発性関節炎または関節痛、妊娠中の感染では自然流産や胎児水腫または死産を引き起こす場合がある。鎌状赤血球貧血などの潜在的な赤血球異常のある患者の場合は重度の無形成発作を起こす場合があり、感染が持続すると慢性骨髄不全となる場合や、免疫不全患者では慢性貧血となる場合がある。文献の報告によると、長期的に血液凝固因子系製剤による治療を受けた患者の体内では B19 の抗体が顕著に上昇する。血液凝固因子製剤中のウイルス含量が 10^8geq/mL を超えると生体は抗体を産生するが、ウイルス含量が 103.5geq/mL の場合には抗体は産生されない。製造用プール血漿の約 60%は 10^2～10^8geq/mL のウイルスを含んでいる。洗浄剤/変性剤 (S/D) 処理を経た血漿でもまだ 23%に B19 が含まれており、ウイルス力価も 10^6～10^8geq/mL と高い一方、プール血漿中の IgG 力価は約 44IU/mL で、抗原を中和するには足りない。現在、米国食品医薬品局 (FDA) では、プール血漿に対しては必ず B19 DNA の検査を行い、B19 DNA の濃度が 10^4geq/mL を超えるプール血漿は廃棄するよう規定している。
本稿では原料血漿および血液製剤に対し B19 DNA の検査と分析を行い、中国人献血者および血液製剤中の B19 DNA の感染状況および汚染状況を調べた。

1.　材料および方法
1.1　試　　料
合格献血者の個人血漿検体 6505 名分は血液製剤製造企業 3 社から調達した。検体はいずれも麦藁色の清澄な液体で、乳糜・フィブリンの析出および溶血、異物がないものであり、それらの採取、輸送および保存の過程はいずれも『中国薬局方』三部 (2010 年版) の血液製剤製造用人血漿に関する規程の要求を満たした。製造用プール血漿 108 検体は上述の血液製剤製造企業 3 社より調達し、-30℃で保存した。静注用人免疫グロブリン 84 ロットは中国企業 20 社から、人フィブリノゲン 24 ロットは中国企業 4 社から、人血液凝固第Ⅷ因子 56 ロットは中国企業 3 社から、人プロトロンビン複合体 33 ロットは中国企業 3 社からそれぞれ調達したもので、いずれも国の検査・審査に合格した製品とした。
1.2　主な試薬および装置
B19 の核酸増幅 (PCR) 蛍光定量検出用試薬キットは中山大学達安基因股份有限公司から購入した。蛍光定量 PCR 装置は米国 ABI 社から購入した。
1.3　B19 DNA の検出
1.3.1　PCR プライマー・プローブ：
位置は B19 ゲノムのコーディング領域の 2000～2300bp 間とした。B19 核酸増幅 (PCR) 蛍光定量検出用試薬キット付属のものを用いた。
1.3.2　ウイルス核酸 DNA の抽出：
滅菌エッペンドルフチューブに溶解バッファー100μL を入れ、さらに検査用検体、試薬キット中の陰性対照および陽

使用上の注意記載状況
その他参考事項等

1. 慎重投与
(1)略
(2)略
(3)略
(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。〕

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性対照 100μL ずつをそれぞれ加え、十分に混和した後、100℃で 10min 処理し、12000r/min で 5min 遠心分離して上清を回収し、保存した。

1.3.3　PCR 増幅：

1.3.2 項の上清 2μL を取り、8000r/min で 10s 遠心分離した。試薬キットの説明書を参照し、PCR 増幅を行った。93℃で 2min の後、93℃/45s、55℃/1min を 10 サイクル、93℃/30s、55℃/45s を 30 サイクル実施し、55℃で 45s 実施した。

1.3.4　結果判定：

55℃のアニーリング期に蛍光シグナルを読み取り、CT 値に基づいて結果を判定した。ROC 曲線法を利用して本試薬キットの参考値を 27 に決定した。蛍光シグナルの増幅が明瞭で、典型的な S 字型の増幅曲線を呈し、CT 値≦27 であれば陽性と判定した。蛍光シグナルに増幅がなく、典型的な S 字型の増幅曲線が見られない場合は陰性と判定した。蛍光シグナルの増幅が明瞭で、典型的な S 字型の増幅曲線が見られるが、CT 値＞27 の場合はグレーゾーンとして再検査を行い、陽性判定基準に適合するかグレーゾーンのままであれば陽性、陰性判定基準に適合すれば陰性と判定した。

2.　結　　果

蛍光定量 PCR の結果、個人血漿、製造用プール血漿、静注用人免疫グロブリン、人フィブリノゲン、人血液凝固第Ⅷ因子、人プロトロンビン複合体中の B19 DNA の陽性数はそれぞれ 6、89、0、11、38、26 であり、陽性率はそれぞれ 0.092%、82.41%、0、45.83%、67.86%、78.79%だった。

3. 考　　察

血液製剤は主に献血者の選択、血漿スクリーニング、プール血漿の検査、製造工程でのウイルス不活化または除去等の手順によって安全性を確保する。現時点ですべての血液製剤の製造工程には 1 段階または複数段階のウイルス不活化または除去手順が含まれる。エンベロープを有するウイルス（HIV、HCV、HBV など）に対しては、現行の原料血漿製造規程の要求事項および製造工程で基本的に製品の安全性を確保できるが、ノンエンベロープウイルス、特に B19 については現行の製造工程での不活化または除去方法の効果が十分とは言えない。欧米等の国ではすでに B19 を原料血漿の一般検査の範囲に含めていることから、中国でもできるだけ早期に原料血漿の B19 の検査方法を確立し、原料血漿と製造用プール血漿に対する検査を行うことで血液製剤の安全性を確保するとともに、国際的な水準に合わせることが必要である。

本試験ではリアルタイム蛍光定量 PCR 法を用いて原料血漿および血液製剤の B19 ゲノム DNA の検査を行った。その結果、中国の血液製剤中の B19 汚染率は海外の文献の報告に比べてわずかに低かった。本試験の結果から、血液凝固因子系製剤の B19 DNA の陽性率がグロブリン系製剤に比べて顕著に高いことが分かったが、その主な原因は血液凝固因子系製剤の製造工程が単純で、通常は簡単な吸着・溶出のプロセスを経るだけであり、かつ製品の純度が低く、ウイルスの除去が不十分なためと考えられる。一方グロブリン系製剤は分離精製の工程が複雑で、何段階ものエタノール沈殿分離およびクロマトグラフィー工程を経てウイルスの大部分は除去される。また、グロブリンの分離に用いる高濃度エタノールも、ウイルスに対し一定の殺滅または破壊作用がある。

現在の中国の PCR 試薬キット性能評価の結果によると、本試薬キットの感度は 1.0×10^4copies/mL であり、検体の検査結果が陰性であることは B19 DNA の濃度が試薬キットの感度を下回ったことを示しているにすぎない。また検体の採取、処理、輸送、保存および試験操作の過程もすべて検査結果に影響を与える可能性があるため、原料血漿採取規程の要求事項に厳格に従って検体の採取と保存を行い、さらに試薬キットの要求事項にも厳格に従って試験操作を行うことで、偽陽性および偽陰性の結果を減少させなければならない。

蛍光定量 PCR 検査はウイルス感染の判定において重要な意義を持っており、中国の血液製剤に対する検査で血液凝固因子系製剤の B19 汚染率が比較的高いことが明らかとなった。しかし現時点で血液凝固因子系の血液製剤を使用後に B19 に感染したとの

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	報告はまだない。その理由の一つとして、多くの患者は体内に B19 に対する中和抗体を持っており、それが比較的良好な保護作用を果たしている可能性がある。またプール血漿中にも大量の B19 中和抗体が含まれ、それも直接的な中和作用を果たすと考えられる。もう一つは、B19 感染は特定の人々にのみ重篤な症状を引き起こすものであり、かつ血液製剤中のウイルス量が多い場合にのみ感染をもたらすが、ウイルス量と感染率の相関性についてはまだ報告がなされていない。さらに、本試験で使用した PCR 法で検出されたものは B19 の DNA であり、生きたウイルスではないという可能性もある。製造工程で精製およびウイルス不活化を経ることで、製品中の潜在ウイルスの大部分は除去または不活化され、感染性を失う。上記を総括すると、B19 の検査方法および血液製剤中の B19 感染状況について今後さらに研究を行い、製品品質を確保する必要があると言える。	

報告企業の意見	今後の対応
ヒトパルボウイルス B19（human parvovirus B19：B19）は、脂質エンベロープを持たない極めて小さな（約 20～26nm）DNA ウイルスで、輸血や血漿分画製剤による伝播が報告されている。他のウイルスに比べて、血漿分画製剤の製造工程での不活化・除去が困難であり、本ウイルスの伝播リスクを完全に否定することはできないため、1996 年 11 月より、使用上の注意に B19 についての記載を行い注意喚起を図ってきた。また、原料血漿への B19 混入量低減のため、日本赤十字社では CLEIA 法を用いたドナースクリーニングが行われている。万一、原料血漿に B19 が混入したとしても、CPV をモデルウイルスとしたウイルスクリアランス試験成績及び B19 を用いた不活化･除去試験の結果から、本剤の製造工程において不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

中国生物制品学杂志 2012 年 8 月第 25 卷第 8 期 Chin J Biologicals August 2012, Vol. 25 No. 8

·技术方法·

原料血浆及血液制品中人细小病毒 B19 DNA 的检测

侯继锋，王敏，马秋平

【摘要】 目的 检测我国原料血浆及血液制品中人细小病毒 B19(Human parvovirus B19)的污染情况。方法 在 B19 基因组编码区的高度保守区 2 000～2 300 bp 之间设计 PCR 引物探针，采用荧光定量 PCR 法检测单人份血浆、生产用混合血浆、静注人免疫球蛋白、人纤维蛋白原、人凝血因子Ⅷ及人凝血酶原复合物中的 B19 病毒 DNA。结果 单人份血浆、生产用混合血浆、静注人免疫球蛋白、人纤维蛋白原、人凝血因子Ⅷ和人凝血酶原复合物的 B19 病毒 DNA 阳性率分别为 0.092%、82.41%、0、45.83%、67.86% 和 78.79%。结论 我国原料血浆及血液制品中 B19 病毒的污染情况略低于国外文献报道，可能与试剂盒的灵敏度及样本量有关，有必要对国内的相关制品作进一步的跟踪。

【关键词】 细小病毒 B19，人；荧光定量 PCR；血液制品

【中国图书分类号】R457.1＋4 R373.9【文献标识码】A 【文章编号】1004-5503(2012)08-1043-03

Determination of human parvovirus B19 DNA in source plasma and blood products

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【Abstract】 **Objective** To determine the contamination with human parvovirus B19 in source plasma and blood products in China. **Methods** Primers were designed based on the highly conserved sequence between 2 000 and 2 300 bp in the coding regions of B19 genome, with which individual human plasma samples, plasma pools for production, intravenous immunoglobulin, human fibrinogen, human coagulation factor Ⅷ and human prothrombin complex were dete rmined for B19 DNA by fluorescent quantitative PCR. **Results** The positive rates of B19 DNA in individual human plasma samples, plasma pools for production, intravenous immunoglobulin, human fibrinogen, human coagulation factor Ⅷ and human prothrombin complex were 0.092%, 82.41%, 0, 45.83%, 67.86% and 78.79% respectively. **Conclusion** The positive rates of B19 virus in source plasma and blood products in China were slightly lower than those reported in the documents abroad, which might be associated with the sensitivity of kit and the quantity of samples. It is necessary to follow the relative message of relevant domestic blood products.

【Key words】 Human parvovirus B19, human; Fluorescent quantitative PCR; Blood products

人细小病毒 B19(Human parvovirus B19)是已知唯一对人类致病的细小病毒[1]。因个体年龄和免疫状态不同，B19 病毒感染后，其临床表现也不相同，儿童感染可引起传染性红斑、成年妇女感染可引起短暂的多关节炎或关节痛、孕期感染可引起自然流产和胎儿水肿或死胎、潜在的红细胞缺陷如镰刀型细胞贫血患者感染可能引起严重的再生障碍危象、持续感染可导致慢性骨髓障碍、而免疫缺陷患者会导致慢性贫血[2-4]。据文献报道[5]，长期接受凝血因子类产品治疗的患者体内抗 B19 病毒抗体显著升高。凝血因子产品中病毒含量大于 10^8 geq/ml 时，机体可产生抗体、病毒含量小于 103.5 geq/ml 时，机体并不产生抗体。约 60%的生产用混合血浆含 10^2～10^8 geq/ml 病毒[6]。经去污剂/变性剂(S/D)处理后的血浆仍有 23%含 B19 病毒，病毒滴度高达 10^6～10^8 geq/ml，而混合血浆中 IgG 滴度约为 44 IU/ml，不足以中和抗原[7]。目前，美国食品药品监督管理局(FDA)已规定，混合血浆应进行 B19 病毒 DNA 检测，B19 病毒 DNA 浓度超过 10^4 geq/ml 的混合血浆应废弃[8]。

本文对原料血浆及血液制品进行 B19 病毒 DNA 检测和分析，以了解中国献血员和血液制品中 B19 病毒的感染和污染情况。

1. 材料与方法

1.1 样品

合格献血员单人份血浆样本 6 505 份来自 3 家血液制品公司，样本均为稻黄色澄清液体，无乳糜和纤维蛋白析出，无溶血，无异物，样本的采集、运输及保存均符合《中国药典》三部(2010 版)的血液制品生产用人血浆规程要求；生产用混合血浆样本 108 份来自上述 3 家血液制品公司，-30℃储存；静注人免疫球蛋白 84 批来自 20 家国内企业；人纤维蛋白原 24 批来自 4 家国内企业；人凝血因子Ⅷ 56 批来自 3 家国内企业；人凝血酶原复合物 33 批来自 3 家

基金项目：国家科技支撑计划项目(2008BAI54B08)。

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国内企业，均为批签发合格制品。

1.2 主要试剂及仪器

B19 病毒核酸扩增(PCR)荧光定量检测试剂盒购自中山大学达安基因股份有限公司；荧光定量 PCR 仪购自美国 ABI 公司。

1.3 B19 病毒 DNA 的检测

1.3.1 PCR 引物探针：位于 B19 基因组编码区 2 000～2 300 bp 之间，由 B19 病毒核酸扩增(PCR)荧光定量检测试剂盒自带。

1.3.2 病毒核酸 DNA 的提取：在灭菌 Eppendorf 管中加入 100 μl 裂解液，再分别加入待检样品、试剂盒自带的阴性对照及阳性对照各 100 μl，充分混匀后，100℃处理 10 min，12 000 r/min 离心 5 min，收集上清，备用。

1.3.3 PCR 扩增：取 1.3.2 项上清液 2 μl，8 000 r/min 离心 10 s。参照试剂盒说明书进行 PCR 扩增：93℃ 2 min；93℃ 45 s，55℃ 1 min，10 个循环；93℃ 30 s，55℃ 45 s，30 个循环；55℃ 45 s。

1.3.4 结果判定：55℃退火阶段收集荧光信号，并根据 *CT* 值判定结果。利用 ROC 曲线法确定本试剂盒的参考值为 27。荧光信号增幅明显，呈典型 S 型扩增曲线，*CT* 值 ≤ 27，判为阳性；荧光信号无增长，无典型 S 型扩增曲线，判为阴性；荧光信号增幅明显，呈典型 S 型扩增曲线，但 *CT* 值 > 27，处于灰度区的样本，经复检，符合阳性判定标准或仍处于灰度区均判为阳性，符合阴性判定标准判为阴性。

2. 结果

经荧光定量 PCR 检测，单人份血浆、生产用混合血浆、静注人免疫球蛋白、人纤维蛋白原、人凝血因子Ⅷ及人凝血酶原复合物的 B19 病毒 DNA 阳性数分别为 6、89、0、11、38 和 26，阳性率分别为 0.092%、82.41%、0、45.83%、67.86% 和 78.79%。

3. 讨论

血液制品主要通过献血员选择、血浆筛查、混合血浆检测、生产工艺病毒灭活或去除等步骤确保其安全性[9]。到目前为止，所有血液制品生产工艺中均含一步或多步病毒灭活或去除步骤。对脂胞膜病毒(如 HIV、HCV、HBV)而言，现行的原料血浆生产规程要求和生产工艺基本能确保制品的安全性；但对于非脂胞膜病毒，尤其是人细小病毒 B19，现行生产工艺中的灭活或去除病毒方法的效果并不理想。欧美等国家已将 B19 病毒纳入原料血浆的常规检测范围。因此，我国有必要尽快建立原料血浆 B19 病毒检测方法，对原料血浆和生产用混合血浆进行检测，以确保血液制品的安全性，并与国际接轨。

本实验采用实时荧光定量 PCR 法对原料血浆及血液制品进行了 B19 病毒基因组 DNA 检测，检测结果显示，国内的血液制品中 B19 病毒污染率略低于国外文献报道[7]，由本实验研究结果可知，凝血因子类产品 B19 病毒 DNA 阳性率明显高于球蛋白类产品，其主要原因可能是，凝血因子类产品工艺简单，通常只需经过简单的吸附和洗脱过程，且产品纯度低，病毒去除不充分。而球蛋白类产品分离纯化工艺复杂，经多步酒精沉淀分离及层析工艺后，大部分病毒已去除。另外，用于球蛋白分离的高浓度酒精对病毒有一定的杀灭或破坏作用。

根据目前国内 PCR 试剂盒产品性能的评估结果，本试剂盒灵敏度为 1.0×10^4 copies/ml，样本检测阴性结果仅表明 B19 病毒 DNA 浓度低于试剂盒的灵敏度，且样本收集、处理、运送、保存及实验操作过程均会对检测结果造成影响。因此，应严格按原料血浆采集规程要求采集并保存样品，并严格按试剂盒要求进行试验操作，以减少假阳性和假阴性结果。

荧光定量 PCR 检测在判定病毒感染方面具有重要意义，通过对国内血液制品检测发现，凝血因子类产品具有较高的 B19 病毒污染率。但目前尚未见输入凝血因子类血液制品后感染 B19 病毒的报道。其原因一方面可能是多数患者自身体内含 B19 病毒中和抗体，中和了进入体内的 B19 病毒，起到了较好的保护作用。同时，混合血浆中含有大量的 B19 病毒中和抗体，有直接的中和作用。另一方面，B19 病毒感染只针对特殊人群引起较严重的症状，且血液制品中病毒载量较高时才会引发感染，而针对病毒载量与感染率之间的相关性尚未见相关报道。其次，可能是本实验使用 PCR 法检测的是 B19 病毒 DNA，而非活病毒。经过生产工艺纯化和病毒灭活后，产品中的大部分潜在病毒均已去除或灭活，无感染性。综上所述，还需对 B19 病毒的检测方法及血液制品中的 B19 病毒感染情况作进一步的研究，以确保产品质量。

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3. 讨论

目前对副结核病尚无有效的治疗方法，根除此病的唯一办法是检出、隔离或淘汰病畜。随着我国国民生活水平的不断提高，牛奶需求呈指数增长，急需大量优质、高产奶牛，因此迫切需要建立早期的快速、准确的副结核病诊断方法，以预防和清除该病，确保我国畜牧业的健康快速发展。

PCR 方法具有高于常规细菌培养的敏感性，并且快速、准确，可及早诊断副结核病，对于该病的控制具有十分重要的应用价值，是目前诊断副结核病的最为理想的方法。

通过添加标准菌株的检测显示，本方法能够较好地从牛乳中检测到副结核分枝杆菌的基因片段。敏感性检测结果显示，本方法的敏感性为 10^2 个细菌/ml，特异性检测结果显示，除含副结核分枝杆菌的乳样为阳性外，含其他细菌的乳样均为阴性。套式PCR 检测方法可很好地用于牛乳中副结核分枝杆菌的检测。本实验方法填补了应用套式 PCR 方法快速检测、检验副结核病的空白。

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(收稿日期：2011-12-23)

医薬品
医薬部外品　　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2012 年 10 月 9 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①② 抗 HBs 人免疫グロブリン		研究報告の公表状況	transfusionnews.com/ 2012/09/28	公表国 ウガンダ	
販売名 (企業名)	① 抗 HBs 人免疫グロブリン筋注 200 単位/1mL「日赤」 (日本血液製剤機構) ② 抗 HBs 人免疫グロブリン筋注 1000 単位/5mL「日赤」 (日本血液製剤機構)					

研究報告の概要

ビデオ：致死率増加に関係したヒトヘルペスウイルス 8 陽性血輸血

近年のウガンダの調査研究によると、ヒトヘルペスウイルス 8 抗体陽性血の輸血は、致死率のリスク増加に関係している。ヒトヘルペスウイルス 8 または HHV-8 は、カポジ肉腫関連ヘルペスとしても知られる。カポジ肉腫、リンパ腫などの病気の原因とされている。HHV-8 は、最初に中東、地中海、アフリカで発見された。サハラ以南のアフリカのいくつかの地域では、陽性率が 50%を超え、急性感染の影響はほとんど知られていない。

ウガンダのカンパラにある病院で 1000 名以上のレシピエントにより、HHV-8 が輸血を通して感染するかどうかの調査が行われた。この調査を解析することにより、研究者は、HHV-8 抗体陽性血を輸血された患者と抗体陰性の血液を輸血された患者の間で 6 か月間、死亡に関するリスクの比較を行った。研究者は、さらに血液の短期間保管について、4 日間とほとんど保管を行わない場合の致死リスクへの影響についても評価を行った。研究は、Journal of Infectious Disease に掲載されている。

研究者らは、HHV-8 抗体陰性血を輸血された場合の死亡率は 7.9%、4 日間以下の保管 HHV-8 陽性血を輸血された患者の場合の死亡率は 17%であった。さらに、短い保管期間の HHV-8 抗体陽性血を輸血された患者は、HHV-8 抗体陰性の血液を輸血された患者の 2 倍近い死亡率であった。

研究者らは、また、短い保管期間の HHV-8 抗体陽性血を受血された患者は、仮に輸血を受けなかった場合よりも 1.8 倍の死亡率であった。HHV-8 抗体陽性の保管期間がなかったものを輸血された患者と HHV-8 抗体陰性の血液を輸血された患者の間には、致死的なリスクの明らかな差は認められなかった。

疾病対策予防センターの Wilfgang Hladik 博士が率いる研究チームは、短い期間保管された HHV-8 抗体陽性の血液の輸血による致死的なリスク上昇の理由についてはまだ明らかにしていない。

Wilfgang Hladik 博士のコメント

「我々は、輸血における死亡リスク上昇は、HHV-8 感染が関連した輸血または、その他の要因によるかどうかまだわかっていない。いずれにせよ、結果からこのような患者の死亡原因の解明のために更なる研究が必要であり、照射血や白血球除去のような潜在的な効果について試験を行う必要がある。」

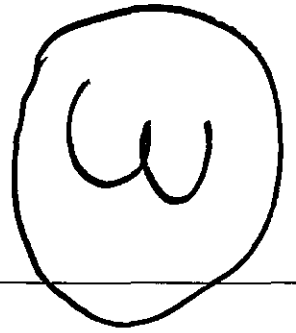
附随の論説の中で、Eva Operskalski 博士は、この研究の結論の解説と潜在的な解説について述べた。Eva Operskalski 博士は、短い期間保管された HHV-8 抗体陽性血に関連した致死的なリスクと輸血量に関係があると指摘している。いずれにせよ、同時に起きているサイトメガロウイルス感染と輸血が関連した免疫抑制の解明にも寄与するかもしれない。

また博士は、輸血の原料が制限された中で、ドナー排除以外のリスク低減の方法を検討するのは不可能に近いだろう。これらの背景から輸血指針は、輸血による感染症の潜在的な可能性のある抗体陽性ドナーの排除によりドナーを減らす効果について評価する必要があると指摘している。HHV-8 抗体陽性血の輸血に関連した作用の更なる研究が必要であろう。

使用上の注意記載状況・その他参考事項等

抗 HBs 人免疫グロブリン筋注 200 単位/1mL「日赤」
抗 HBs 人免疫グロブリン筋注 1000 単位/5mL「日赤」

血液を原料とすることに由来する感染症伝播等
vCJD等の伝播のリスク



医薬品
医薬部外品　　研究報告　調査報告書
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我々のコメントは 10 月 15 日付の Transfusion News の他の項に掲載予定である。しばらくの間は、常に transfusionnew.com で最新情報の収集を行うことができます。		
報告企業の意見	今後の対応	
ヘルペスウイルスは、直径120～200nmの球状のエンベロープを有する2本鎖のDNAウイルスで、ヒトヘルペスウイルス8（human herpesvirus 8：HHV-8、別名：カポジ肉腫関連ヘルペスウイルス）はガンマヘルペスウイルス亜科ラジノウイルス（rhadinovirus）属に属する。万一、原料血漿にHHV-8が混入したとしても、BHVをモデルウイルスとしたウイルスクリアランス試験成績から、本剤の製造工程において不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。	

VIDEO: Human Herpesvirus 8 Seropositive Blood Transfusion Is Associated with Increased Mortality

A recent study from Uganda demonstrated that transfusion with human herpesvirus 8 antibody-positive blood is associated with an increased risk of mortality. Human herpesvirus 8, or HHV-8, is also known as Kaposi's sarcoma-associated herpesvirus. It can cause Kaposi's sarcoma, lymphoma, and other diseases. HHV-8 is found primarily in the Middle East, the Mediterranean, and Africa. Seroprevalence is greater than 50% in some areas of sub-Saharan Africa, but little is known about the impact of acute infections.

Using a cohort of more than 1000 transfusion recipients in a hospital in Kampala, Uganda, it had previously been demonstrated that HHV-8 could be transmitted through blood transfusions. By analyzing data from this cohort, researchers compared the risk of death within six months of transfusion between patients who received HHV-8 antibody-positive blood and patients who received antibody-negative blood. The researchers also assessed the additional impact of the short-storage – or storage for four or fewer days – on mortality risk. The study was published in the *Journal of Infectious Diseases.*

The researchers found that while 7.9% of patients who received HHV-8 antibody negative blood died, 17% of patients who received HHV-8 antibody-positive blood stored for four days or less died. Furthermore, transfusion patients who received short-stored HHV-8 antibody-positive blood were about twice as likely to die as patients who had received HHV-8 antibody-negative blood.

The researchers also found that for each additional unit of short-stored HHV-8 antibody-positive blood received, patients were 1.8 times more likely to die than if they had not received the unit. Patients who received additional units of HHV-8 antibody-positive blood that had not been short-stored or additional units of HHV-8 antibody-negative blood, did not face a significantly elevated risk of mortality.

The research team, led by Dr. Wolfgang Hladik of the Centers for Disease Control and Prevention, noted that the reasons for the increased mortality risk associated with short-storage of HHV-8 antibody-positive blood transfusions are still unclear.

Here is Dr. Hladik.

"We don't know whether the higher risk of death following transfusion was due to transfusion-associated HHV-8 infection, or some other factor. However, the results support the need for more research to determine the cause of death in such patients, and to examine the effect of potential interventions such as irradiation or leukoreduction."

In an accompanying editorial, Dr. Eva Operskalski discussed potential explanations for and implications of this study's findings. Dr. Operskalski noted that there appeared to be a dose-response relationship for the mortality risk associated with

short-stored HHV-8 seropositive blood. However, that concurrent infection with cytomegalovirus and transfusion-associated immunosuppression may have contributed as well.

She also noted that in resource-limited settings, it may not be possible to pursue risk reduction methods other than donor exclusion. Transfusion policy in these settings would need to weigh the benefits of reducing the donor pool by excluding seropositive donors with the potential for transfusion-transmitted infections. Further studies of the mechanisms behind the association of transfusion with HHV-8 seropositive blood are needed.

We'll be back on October 15 with another edition of Transfusion News. In the meantime, you can always keep up to date with all of the latest information by visiting transfusionnews.com. Thanks for joining us.

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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	新医薬品等の区分	総合機構処理欄
				2012. 9. 19	該当なし	

一般的名称	解凍人赤血球濃厚液	研究報告の公表状況	Benjamin RJ, Stramer SL, Leiby DA, Dodd RY, Fearon M, Castro E. Transfusion. 2012 Sep;52(9):1913-21; quiz 1912. doi: 10.1111/j.1537-2995.2011.03554.x. Epub 2012 Feb 10.	公表国 米国・カナダ・スペイン
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社) 解凍赤血球液-LR「日赤」(日本赤十字社) 照射解凍赤血球液-LR「日赤」(日本赤十字社)			

研究報告の概要

○北米とスペインの*Trypanosoma cruzi*感染症:輸血感染を支持する証拠

背景: 米国、カナダ及びスペインは、輸血感染を防ぐために*Trypanosoma cruzi*(*T.cruzi*)感染症(シャーガス病)の選択的供血者検査を行っている。輸血感染症に関連する供血者、製剤及び患者の特性が再調査され、感染の血清学的所見を持つ供血者由来血液成分の感染性が推定された。

研究デザイン及び方法: 輸血感染*T.cruzi*症例の体系的レビュー及び北米及びスペインで実行された受血者追跡事例より、輸血感染の証拠について評価された。

結果: 20人の受血者における*T.cruzi*感染症は、1987年～2011年の期間中の血清学的感染確認供血者18人(受血者追跡のみによって確認された11人を含む)に関連していた。症例は地理的に広く分布していて、偶発感染または土着感染との関連は見られず、免疫不全状態の患者のみに症状が見られた。感染が特定された患者は全て白血球除去及び照射製剤を含むアフェレーシスまたは全血由来の血小板製剤による感染であった(n=11)。全血輸血に感染の可能性はあるが、赤血球及び凍結製剤による感染の証拠はない。受血者追跡によって、抗体陽性供血者からの血液成分の感染性は全体で1.7%(95% CI、0.7%-3.5%)と低いことが明らかとなった(血小板13.3%:95% CI、5.6%-25.7%、赤血球0.0%:95% CI、0.0%-1.5%、血漿及びクリオプレシピテート0.0%:95% CI、0%-3.7%)。

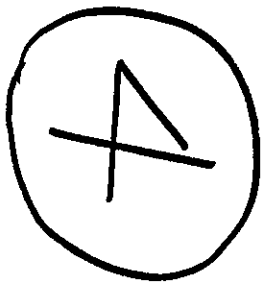
結論: *T.cruzi*は抗体陽性の一部の供血者からの血小板製剤により感染する。調査以前の感染についての証拠は希薄であり、血小板及び新鮮全血供血において選択的検査が考慮されるべきである。

使用上の注意記載状況・その他参考事項等

解凍赤血球濃厚液「日赤」
照射解凍赤血球濃厚液「日赤」
解凍赤血球-LR「日赤」
照射解凍赤血球-LR「日赤」
解凍赤血球液-LR「日赤」
照射解凍赤血球液-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
輸血*T.cruzi*感染症に関連する供血者、製剤及び患者の特性を再調査し、抗体陽性供血者の血液成分の感染性を評価したところ、明確な輸血*T.cruzi*感染はアフェレーシスまたは全血由来の血小板製剤に関連し、赤血球及び凍結製剤による感染の証拠はなかったとの報告である。	日本赤十字社は、輸血感染症対策として献血時に海外滞在歴の有無を確認し、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、厚生労働科学研究「血液製剤の安全性確保と安定供給のための新興・再興感染症の研究」班と共同して検討している。新たに中南米出身者(母親が出身を含む)、通算4週間以上の中南米滞在歴を有する献血者からの血液は、血漿分画製剤の原料のみ使用する対策を実施することとした。今後も引き続き情報の収集に努める。



CME

Trypanosoma cruzi infection in North America and Spain: evidence in support of transfusion transmission

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BACKGROUND: The United States, Canada, and Spain perform selective testing of blood donors for *Trypanosoma cruzi* infection (Chagas disease) to prevent transfusion transmission. The donor, product, and patient characteristics associated with transfusion-transmitted infections are reviewed and the infectivity of components from donors with serologic evidence of infection is estimated.

STUDY DESIGN AND METHODS: A systematic review of transfusion-transmitted *T. cruzi* cases and recipient tracing undertaken in North America and Spain is described. Cases were assessed for the imputability of the evidence for transfusion transmission.

RESULTS: *T. cruzi* infection in 20 transfusion recipients was linked to 18 serologically confirmed donors between 1987 and 2011, including 11 identified only by recipient tracing. Cases were geographically widely distributed and were not associated with incident or autochthonous infections. Index clinical cases were described only in immunocompromised patients. All definite transmissions (n = 11) implicated apheresis or whole blood–derived platelets (PLTs), including leukoreduced and irradiated products. There is no evidence of transmission by red blood cells (RBCs) or frozen products, while transmission by whole blood transfusion remains a possibility. Recipient tracing reveals low component infectivity from serologically confirmed, infected donors of 1.7% (95% confidence interval [CI], 0.7%-3.5%) overall: 13.3% (95% CI, 5.6%-25.7%) for PLTs, 0.0% (95% CI, 0.0%-1.5%) for RBCs, and 0.0% (95% CI, 0%-3.7%) for plasma and cryoprecipitate.

CONCLUSIONS: *T. cruzi* is transmitted by PLT components from some donors with serologic evidence of infection. Evidence of transmission before the implementation of widespread testing in the countries studied is sparse, and selective testing of only PLT and fresh whole blood donations should be considered.

Universal serologic testing of US blood donors for *T. cruzi* infection was initiated by the two largest blood collecting systems, the American Red Cross (ARC) and Blood Systems, Inc., in early 2007, after the US Food and Drug Administration's (FDA's) approval of a screening assay in December 2006 and a recommendation by the FDA's Blood Products Advisory Committee (BPAC).[1] The decision to implement blood donation screening was based on accumulated evidence that a substantial number of donors nationwide had evidence of prior exposure; reported transmission rates of 12% to 20% based on historical findings from South America; and case reports of transfusion transmissions in the United States, Canada, and Spain.[2-17] FDA draft guidance recommending universal blood donation screening was released for comment in March 2009. After 16 months of testing, serologic evidence of infection was confirmed in approximately 1:27,500 donors overall but was especially concentrated in areas with large Latin American immigrant communities.[1,18] With an observed low rate of transfusion transmission and apparent absence of incident infections in the US donor pool, many blood centers moved to selective one-time testing of all donors.[19-21] Final FDA guidance released in December 2010 endorsed this approach. In September 2005, Spain implemented selective qualification by testing or

ABBREVIATIONS: ARC = American Red Cross; BPAC = Blood Products Advisory Committee.

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Received for publication November 5, 2011; revision received December 19, 2011, and accepted December 19, 2011.

doi: 10.1111/j.1537-2995.2011.03554.x

TRANSFUSION 2012;52:1913-1921.

exclusion of donors who revealed histories during donor questioning suggesting *T. cruzi* infection risk, after documentation of transfusion-transmitted cases implicating immigrant donors from South America.[22] Both blood providers in Canada implemented similar selective testing models in 2009 to 2010.[23]

After more than 4 years of screening for *T. cruzi* infection in blood donors and recipient tracing of prior donations from serologically confirmed-positive donors, it is timely to reexamine the evidence for transfusion-transmitted *T. cruzi* outside of endemic areas to evaluate the impact of testing on patient safety. It is especially important to assess the imputability of reported cases, as many are described only in case reports of varying quality and completeness. Therefore, we embarked on a systematic review of published reports of transfusion-transmitted *T. cruzi* and of recipient tracing investigations, to assess the characteristics of the donors, patients, and products involved.

MATERIALS AND METHODS

Publication inclusion criteria

This review seeks to summarize all reported cases of transfusion-transmitted *T. cruzi* in North America (Canada, United States, and Mexico) and Spain, with an evaluation of imputability to assess the reliability of each report. Cases were diagnosed either as index clinical infections that were subsequently linked to transfusion or through identification of infected blood donors by serologic screening and tracing of recipients of blood products derived from earlier donations from those donors (recipient tracing). Reports were obtained by literature review and PubMed searches; however, some cases are reported in abstract form only and the authors relied on personal communications with experts in the United States and Spain to assist in identifying reports. A PubMed search between January 1980 and August 2011 on the terms "*Trypanosoma cruzi*" and "transfusion" revealed 377 abstracts that were reviewed for evidence of transfusion transmission and/or recipient tracing studies occurring in the United States, Mexico, Canada, or Spain. Included are all cases identified in the United States by recipient tracing and reported to the BPAC on August 2, 2011.[24]

Imputability

Each case of suspected transfusion-transmitted *T. cruzi* was assessed for imputability based on the available published data using a classification scheme consistent with the Centers for Disease Control and Prevention's National Healthcare Safety Network criteria.[25] To be considered transmission by transfusion, a case had to describe a patient with a clinical or laboratory diagnosis of *T. cruzi* infection (with or without clinical symptoms) and a history of transfusion. Possible cases included any patient where no donor was identified as a potential source of infection but transfusion transmission was thought likely on epidemiologic grounds, or recipient tracing cases where an infected donor and recipient were identified but recipient infection before transfusion could not be excluded and the infected patient had a major risk factor for prior infection, such as having lived in and/or being born in a *T. cruzi*–endemic area. Probable cases included those in which an infected blood donor was identified through serologic testing, with or without further confirmatory testing, but the patient may have at least one other weak risk factor for prior *T. cruzi* infection such as travel to endemic areas; however, transfusion transmission was thought likely on epidemiologic grounds.[26] For the purposes of this study, definite cases included infected patients with no other recorded risk factors and who were transfused with a blood product from a donor shown to be infected with *T. cruzi*.

Statistical analysis

Infectivity by transfusion is expressed as the percentage of cases identified by recipient tracing and the 95% confidence interval (CI), as determined by the Mid-P Exact method.[27]

RESULTS

Fifteen reports were identified that document suspected *T. cruzi* transfusion transmission in 20 patients, including six in abstract form only (Table 1). Seven reports were from the United States, five reports were from Spain, two from Canada, and one from Mexico. Eleven patient cases met the imputability definition of definite transfusion transmission, with six of these identified only by recipient tracing of which one was linked by genetic analysis of donor and recipient *T. cruzi* isolates.[2-16]

Five definite cases were discovered on clinical grounds, all in immunocompromised patients undergoing chemotherapy for cancer and/or stem cell transplant. Definite cases were widely distributed geographically with three cases in New York; one each in Florida, Rhode Island, and Canada; and five cases in four separate regions of Spain. Distribution did not correlate with areas known to harbor vectors capable of transmitting *T. cruzi* infection. Additionally, there were no cases ascribed to autochthonous infections or recently infected donors. The donors involved were born most commonly in Bolivia (six cases), but others came from Argentina, Brazil, Chile, and Paraguay. All donors involved in US cases were long-term residents of the United States (16-40 years). No implicated donors came from Mexico or Central America. All definite cases implicated transfusion of a platelet (PLT) product,

TABLE 1. Patient, donor, and product characteristics involved in transfusion-transmitted *T. cruzi* infections

Reference, year, recipient location	Recipient condition	Other risk factors	Donor implicated	Donor origin	Products	Transfusion transmission
1. Grant et al., 1989,[11] New York	Hodgkin's disease	No	Yes	Bolivia	PLTs	Definite
2. Leiby et al., 1999,[4] Florida	*Multiple myeloma**	No	Yes	Chile	PLTs (whole blood)	Definite
3. Nickerson et al., 1989,[12] Canada	Acute lymphoblastic leukemia	No	Yes (2)	Paraguay (2)	PLTs	Definite
4. Young et al., 2007,[15] Rhode Island	Neuroblastoma	No	Yes	Bolivia	PLTs (irrad. & leuko.)	Definite
5. Kessler et al., 2010,[8] New York	Unknown*	No	Yes	Argentina	PLTs (irrad. & leuko. apheresis)	Definite
6. Kessler et al., 2010,[8] New York	Unknown*	No	Yes	Argentina	PLTs (leuko. apheresis)	Definite
7. Flores-Chavez et al., 2008,[13] Madrid	Leukemia	No	Yes	Brazil	PLTs	Definite
8. Perez et al., 2008,[5] Malaga	Aplastic anemia	No	Yes	Bolivia	PLTs	Definite
9. Perez et al., 2008,[5] Malaga	Choroid plexus papilloma*	No	Yes	Bolivia	PLTs	Definite
10. Abalo et al., 2007,[7] Galicia	Unknown*	No	Yes	Bolivia	PLTs	Definite
11. Ozaeta Orrono et al., 2008,[9] Basque	Unknown*	No	Yes	Bolivia	PLTs	Definite
12. Lane et al., 2000,[3] Canada	Promyelocytic leukemia	Travel to Mexico	Yes	Paraguay	Not defined (299 PLT units, 8 RBC units)	Probable
13. Geiseler et al., 1987,[6] California	Leukemia	Travel to Mexico	Yes	Not stated	Not defined	Probable
14. Stramer et al., 2008,[20,28] Tennessee	Pregnancy*	Born in El Salvador	Yes	El Salvador	PLTs	Possible
15. Kirchhoff et al., 2006,[16] Mexico	Unknown*	Endemic region	Yes	Mexico	Fresh whole blood or PLTs	Possible
16. Kirchhoff et al., 2006,[16] Mexico	Unknown*	Endemic region	Yes	Mexico	Fresh whole blood or PLTs	Possible
17. Kirchhoff et al., 2006,[16] Mexico	Unknown*	Endemic region	Yes	Mexico	Fresh whole blood or PLTs	Possible
18. Kirchhoff et al., 2006,[16] Mexico	Unknown*	Endemic region	Yes	Mexico	Fresh whole blood or PLTs	Possible
19. Cimo et al., 1993,[10] Texas	Colon cancer	Travel to Mexico	No	No donor identified	~500 RBC, PLT, and plasma units	Possible
20. Villalba et al., 1992,[14] Cordoba	Leukemia	No	No	No donor identified	20 products, not defined	Possible

* Found on recipient tracing.
Irrad. = irradiated; leuko. = leukoreduced.

either from a whole blood or an apheresis donor. In two of these cases, the implicated PLT product was documented to have been both leukoreduced and irradiated[8,15] (D. Kessler, personal communication, 2011). Donations from some of the donors whose PLT products were associated with definite transmissions of *T. cruzi* were subjected to recipient tracing. Overall six definite transmissions were linked by recipient tracing from five donors identified either by donor screening (four) or as an index clinical case (one).[4,5,7-9]

Two cases met the definition of probable transfusion transmission. In both cases, the patient's risk factor involved "travel to Mexico"; recent travel to endemic countries is considered a weak risk factor given improved vector control, along with a requirement for sustained residence in an endemic setting to successfully transmit the parasite.[24,26] Lane and coworkers[3] described a case in Canada in which an implicated donor was born in Germany, but lived extensively in Paraguay. The recipient, who had prolymphocytic leukemia, had received 299 PLT concentrates and eight red blood cell (RBC) units; no specific component was implicated in this transmission case. A second case described by Geiseler and colleagues[6] did not define a component type although a directed donor was implicated by serologic testing (father who had lived in Mexico).

Seven cases were considered to be possible transfusion transmission. The ARC had one possible case associated with a serologically confirmed-positive donor identified by routine blood donation screening. The ARC implemented routine universal screening of all blood donors for *T. cruzi* infection by enzyme-linked immunoassay (EIA; ORTHO *T. cruzi* enzyme-linked immunosorbent assay test system, Ortho Clinical Diagnostics, Raritan NJ) in January 2007, with confirmation of repeat reactivity by radioimmunoprecipitation assay (RIPA; Quest Diagnostics, Chantilly, VA). Recipient tracing was performed for all repeat donors confirmed positive by RIPA.[18,20,28] During the first year of screening, a donor with a history of birth in Brazil donated a whole blood unit to the Tennessee Valley Region Blood Center and was found to be ELISA repeatedly reactive, RIPA positive, but *T. cruzi* PCR and hemoculture negative by in-house methods.[4] Components from four prior whole blood donations were investigated for transmission. The 28-year-old female recipient of a leukoreduced whole blood PLT unit transfused in August 2006 was found to be *T. cruzi* ELISA repeat reactive, RIPA positive, and PCR and hemoculture negative. This recipient had a history of birth in El Salvador, an endemic region for *T. cruzi*.[28] Conclusive evidence of transfusion transmission was not possible through genetic characterization, as parasites could not be recovered from either the donor or the recipient. A prior recipient of a fresh-frozen plasma (FFP) unit was found to be negative for all tests. The other recipients of the donors' blood were either deceased or lost to follow-up. This case meets our definition of a possible transfusion transmission. Since that time and covering 4 years of screening, the ARC has not identified another infected recipient of a blood product from a confirmed-positive donor (110 total recipients tested; however, only nine received PLTs).

Kirchhoff and colleagues[16] describe a serologic screening study in Mexico that revealed an overall 0.75% prevalence of *T. cruzi* infection in blood donors at five regional blood centers. Recipient tracing investigations were performed at one center in Guadalajara where the prevalence in blood donors was 0.79%. Four of nine (44%) recipients of either whole blood (two) or PLTs (two) from infected donors were found to be serologically positive. The authors concluded that transfusion transmission had occurred based on a significant difference between the rate of positive findings in the tested recipients and that in the donor population, of which 83% were less than 35 years old. No further details on the donors or recipients, including the recipients' pretransfusion serologic status, age, and underlying disease, or the prevalence of *T. cruzi* in the general patient population, were provided. Direct demonstration of *T. cruzi* infection by PCR or microscopy was not performed in either the donor or the recipient populations in this study. Thus, evidence for transfusion transmission is regarded as possible in these cases.

Two other cases of suspected *T. cruzi* transmission are reported by Villalba and colleagues[14] in Cordoba, Spain, and by Cimo and colleagues[10] in Houston, Texas, based on the detection of clinical infection in multiply transfused patients with no reported history of other risk factors for infection. However, investigation of the blood donor population was incomplete and no infected donors were identified, leading to the conclusion of possible transfusion transmission.

The relatively sparse number of reports of transfusion-transmitted *T. cruzi* in North America and Spain, and the absence of definite cases implicating transfusions involving blood products other than PLTs, raises a question regarding the relative infectivity of components from infected donors. In addition to the transfusion-transmitted cases identified by recipient tracing described above, at least six other reports of recipient tracing studies have failed to identify additional cases of transfusion transmission.[18,19,21,29-32] In total, it is noteworthy that only six definite transfusion transmission cases have been identified through recipient tracing of 350 recipients, suggesting an overall infectivity of 1.7% (95% CI, 0.7%-3.5%) for recipients exposed to blood components from seropositive donors (Table 2). While the full breakdown of component type is incomplete, available data allow us to calculate the infectivity risk for 0 of 197 RBC units (0.0% [95% CI, 0.0%-1.5%]); 6 of 45 PLTs (13.3% [95% CI, 5.6%-25.7%]); and 0 of 80 frozen plasma or cryoprecipitate units (0.0% [95% CI, 0%-3.7%]).

TABLE 2. Outcomes of successful recipient tracing investigations on prior donations from donors found to be infected with *T. cruzi* either after identification of an index clinical case or by serologic screening of blood donors in the research or routine operations setting

	Transfused and investigated				
Report	Total† components	RBCs	PLTs	Plasma or cryoppt	Whole blood
1. Grant et al., 1989[11]	5				
2. Kerndt et al., 1991[31]	7			1	
3. Leiby et al., 1999[4]	1		1*		
4. Leiby et al., 1999[30]	1		1		
5. Leiby et al., 2002[2]	18	11	2	5	
6. Abalo et al., 2007[7]	2	1	1*		
7. Kessler et al., 2008[9]	4	2	2*		
8. Perez de Pedro et al., 2008[5]	8		1*		
9. Ozaeto Orrono et al., 2008[9]	2	1	1*		
10. Fearon et al., 2011[32]	48	30	6	12	
11. FDA BPAC, 2011[24]	254	152	30‡	62	3
Totals	350	197	45	80	3

* Positive recipient tracing investigations with definite infected recipients identified.
† Total component count does not correspond to the individual component count due to missing data in the referenced publications.
‡ A single possible transfusion transmission case is excluded from this analysis.
Cryoppt = cryoprecipitate.

DISCUSSION

In December 2006, the FDA licensed the first blood screening test for antibodies to *T. cruzi* that has been used with a laboratory-developed test (RIPA) for confirmation of repeat reactivity.[1,33] The BPAC met to discuss the development of guidance for blood donation screening in April 2007. The FDA released draft guidance for universal testing in March 2009 and then final guidance in December 2010 allowing a selective testing model where donors need only test negative one time to be qualified for all future donations. Evidence in favor of blood donation screening includes a significant prevalence of *T. cruzi* seropositivity in blood donors, especially in regions of the United States with substantial Latin American immigrant populations. In the late 1990s prevalence rates were as high as 1 in 7500 in Southern California donors and 1 in 9000 in donors in Miami;[29,30] current data reflect rates of 1 in 6800 and 1 in 5000, respectively (ARC internal data). Furthermore, prior reports from endemic countries suggested that approximately 12% to 20% of components from seropositive donors transmit infection, including whole blood, RBCs, PLTs, white blood cells (WBCs), FFP, and cryoprecipitate.[34] Selective one-time testing of all US blood donors was supported by BPAC and incorporated into final FDA guidance based on the paucity of evidence for incident or autochthonous infections in the United States and the low overall rate of transfusion transmission demonstrated by recipient tracing following the first 4 years of testing.[1,21]

In Spain, a European country with a high immigration rate from South America, a Royal Decree was issued in 2005 whereby the Spanish Ministry of Health required the exclusion or testing of donors born in endemic countries, the children or grandchildren of mothers born in those countries, or donors with a history of having resided or being transfused in those areas.[22,35] Both blood providers in Canada recently implemented similar measures, after the report of two transfusion-transmitted cases.[3,12]

Since January 2007, more than half of the US blood supply, consisting of more than 20 million transfused components derived from approximately 10.9 million donors each year,[36] has been tested. A total of 1456 RIPA–confirmed-positive donors were identified by August 2011, according to the AABB Chagas Biovigilance Network,[37] for a mean rate of approximately 1:25,000 to 1:30,000 donors tested, consistent with prior reports.[18,20,28] Substantially higher rates are apparent in areas of the country with larger immigrant populations from Mexico and South and Central America.

Despite the relatively high prevalence of *T. cruzi* infection in the United States, evidence of transfusion transmission is sparse. Only five cases (three definite, one probable, and one possible transmission case) were reported in the United States between 1987 and 2011, with recipient tracing identifying another two definite and one possible case after the introduction of routine donor testing. It has been suggested that acute *T. cruzi* infection may often be subclinical and that it may only be recognized in highly immunocompromised patients. Thus, transfusion transmission may be significantly underreported.[38] Clinical cases confirm the propensity for diagnosis in immunocompromised patients; however, recipient tracing has failed so far to identify a substantial number of seropositive recipients, recognizing that the number of PLT recipients tested is limited (e.g., only nine over 4 years at the ARC). In any event, it is desirable to reassess the available data in support of *T. cruzi* transfusion transmission and resulting screening policies.

Review of 20 cases derived from 15 reports confirms that there is strong evidence for transfusion transmission; however, all cases where a component was identified involved either PLTs (from both whole blood and apheresis) or whole blood. Leukoreduction and/or irradiation of PLT components do not appear to prevent transmission. The patients detected clinically were immunocompromised and under treatment for oncologic diseases and thus more likely to receive PLT products. Donors involved in transmission cases were invariably born in endemic areas of South and Central America or Mexico and donated blood across the geography of North America or Spain. In the United States and Canada, there is little evidence of increased transmission risk from donations in those areas of the country having the highest proportion of immigrant donors from endemic countries, or from areas at risk for autochthonous transmission, as evidenced by cases in New York, Rhode Island, Manitoba, and Tennessee versus a high concentration of infected donors in California, Florida, and Texas.

There is no evidence in this review of published cases and recipient tracing that plasma, cryoprecipitate, or RBCs transmit *T. cruzi* infection. The evidence for whole blood transmission relies on statistical comparisons of infection rates in donors and patients in Mexico, but transmissibility cannot be excluded. It should be noted that in Mexico, as in much of Latin America, the use of replacement donors remain and the transfusion of "fresh" whole blood within days of collection is much more common than in the United States.

The relative paucity of reports of transmission in Mexico is difficult to understand. *T. cruzi* is endemic in many parts of Mexico and screening of blood donors has revealed a seroprevalence that varies from 0.37% to 7.7%, with a mean of 1.5% in 18 government blood centers in various states.[16,39-44] It seems reasonable to assume that this is due to underreporting and the difficulty in diagnosing transfusion transmission in endemic areas.

The risk of a donor with serologic evidence of *T. cruzi* infection transmitting infection is unknown, but appears to be low. In aggregate, recipient tracing has identified only six confirmed cases in 350 patients transfused and tested, for an overall infectivity rate of 1.7% (95% CI, 0.7%-3.5%). How do we reconcile the data from nonendemic countries suggesting low transmissibility with historical reports of higher transmissibility in endemic countries? It may be that prior estimates are incorrect, that the risk has changed over time, that the risk of transmission varies with different geographical strains of *T. cruzi*, or a combination of the above. First, we must acknowledge the difficulty in assessing transmission in endemic areas. The widely quoted rate of 12% to 20% transmission is based on estimates published by the World Health Organization in 1980 followed by a review of the evidence basis by Schmunis in 1991.[17,38,45]

The early studies often relied on comparisons of infection rates in donor and multiply transfused patient populations as evidence of transmission. The assertion that recipients with higher rates of infection reflected transfusion transmission is likely to be confounded by the fact that infection may have occurred decades ago in rural areas of endemic countries before effective vector control. In addition, because Chagas disease is a chronic infection with long-term sequelae that may require transfusion, and that most blood is transfused to the elderly, infection is more likely to be recognized in transfused patients versus blood donors who are generally much younger.[16,17] These studies cannot confirm infection because there was no pretransfusion sample, which is necessary to prove seroconversion indicative of transmission.[46] In addition, proof of transfusion transmission is especially difficult as serologic conversion may occur 3 or more months after transfusion transmission, requiring PCR testing or hemoculture to make a diagnosis.[4] Thus, the accuracy of early reports of infectivity from endemic areas should be questioned. Indeed, at least one study stated the risk of transmission from a single transfusion is 0.15% to 0.6%, which is consistent with our current observations.[47,48]

Second, the assumed 12% to 20% rate of transmission was established by studies performed before 1980.[17] During that period, the use of paid and/or replacement donors and the transfusion of fresh whole blood or nonleukoreduced blood was more likely than is currently the case. With the move to modern RBC storage solutions, prolonged storage times, and widespread leukoreduction by filtration, the risk of transfusion transmission may have changed over time. Transmission by PLTs shows that *T. cruzi* organisms can survive in anticoagulant solution with storage up to 5 days at room temperature and that leukoreduction and irradiation may reduce but do not eliminate the risk. The absence of transmission by RBCs or noncryopreserved frozen products suggests that the organisms are either removed during whole blood processing and/or do not survive storage or freezing well.[49] *T. cruzi* exists exclusively as an extracellular parasite in the blood and may be particularly susceptible to processing and storage conditions. Dzib and coworkers[50] showed that leukoreduction by centrifugation reduces but does not eliminate *T. cruzi* contamination from RBC, buffy coat, and PLT fractions while Moraes-Souza and colleagues[49,51] showed similar data for leukoreduction by filtration. Alternatively, Hernandez-Becerril and coworkers[41] found that while 41% (12 of 29 donors) of seropositive donors in Mexico City were also positive by PCR and 10% (2 of 29 donors) by hemoculture, suggesting active parasitemia, they were unable to detect any parasitemia in 70 RBC and PLT components prepared from blood donations from similar donors. The authors suggest that processing of whole blood may diminish the parasite burden in blood components by eliminating the WBC-

rich fraction. Given that RBCs are usually transfused after longer storage periods than PLTs, it is entirely possible that the decreased infectivity for RBC products compared to PLTs is due to organism attrition during processing and storage.

Finally, Leiby and colleagues[52] suggest that different strains of *T. cruzi* may pose varying risks of transmission. Most of the immigrant population in Spain where a number of cases of transfusion transmission have been documented is derived from South America where *T. cruzi* (Tc) Lineages II to VI predominate, whereas the immigrant population in the United States, where the rate of transmission is low, is more likely to hail from Mexico and Central America where Tc Lineage I is found. The authors report a significantly higher rate of hemoculture positivity with Tc Lineage II to VI (11 of 24 [45.8%]) versus Tc Lineage I (2 of 90 [2.2%]). Higher levels of parasitemia with Tc Lineages II to VI may explain the higher rates of transmission in countries where immigration from South America predominates. A study conducted by the Centro de Transfusión de Cruz Roja Española en Madrid provides further support for this hypothesis: 15 of 49 (30.6%) blood donors found to be *T. cruzi* antibody positive were also positive by hemoculture, with parasite levels between 1 and 10 parasites/mL. These donors were all born in South America and most were from Bolivia (E. Castro, unpublished data).

Serologic testing for *T. cruzi* adds significantly to the cost of providing blood products to patients while adding little safety benefit, given the sparse evidence for transfusion transmission. For these reasons, Spain and Canada have restricted testing to the small subset of donors who acknowledge risk factors for infection. In US studies, Leiby and colleagues[29] showed that donor country of birth and time in endemic countries were informative donor history determinants, but Custer and colleagues[53] found that they were only able to identify 75% of confirmed *T. cruzi* infections, suggesting that donor history would have limited utility as a safety measure in the United States.

The FDA has released guidance recommending selective donor testing on one occasion, based on the absence of evidence for incident infections in the US donor population.[21] Our analysis now suggests that before any testing, the risk of transfusion transmission was restricted to PLT (and possibly fresh whole blood) transfusions. Selective testing of only these donations would constitute a level of safety that could eliminate any measurable risk of transfusion transmission of *T. cruzi*, while conserving resources for other interventions with higher recipient impact (e.g., *Babesia microti*).

CONFLICT OF INTEREST

None.

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識別番号・報告回数			報告日	第一報入手日 2012 年 11 月 27 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 2012; 11: 1-10	公表国 イギリス	
販売名 (企業名)	ハプトグロビン静注 2000 単位「ベネシス」 (日本血液製剤機構)					

研究報告の概要

緒　　言

伝達性海綿状脳症(TSE)は、ヒトと他の動物の稀な神経変性疾患である。1986 年、英国での牛海綿状脳症(BSE)の発生は、ヒトへの伝播の可能性の点から見て、懸念材料を与えた。これは、変異型クロイツフェルトヤコブ病(vCJD)として知られるこの疾患のヒト型の最初の症例が 1995 年に認識された時、現実であることが分かった。ヒトにおけるこの疾患の現状は、無症候性キャリヤーからのヒトーヒト感染は、血液成分または血漿分画製剤を介して潜在的に起こる可能性を増やす。多数のドナーに由来する血漿分画製剤のためのこの潜在的リスクに対する懸念は、Bio Products Laboratory Ltd(BPL)による分画の英国血漿から米国血漿に 1999 年に切り替えることに英国保健省を導いた。vCJD が国外で起こった症例は別として米国で基本的に起こっていないことが、理論的な伝達リスクを減らす即座の安全措置とみなされた。血漿分画製剤の場合、vCJD 疾患の伝播の証拠を明らかにしていない。しかし、細胞（性）血液製剤による 4 つの伝播の可能性症例があった。その上、vCJD 感染の不顕性症例も、死後の患者の脾臓の中の PrP^{SC} 発見によって確認された；患者は他の危険因子の内、英国血漿由来血漿から作られた精製第Ⅷ因子の中間体（8Y）を投与された。

製造工程は一般に TSE 物質を除去できることを示す数種の血漿分画製剤の有用なデータがある一方、BPL で製造された製品の有用なデータはない。現在の研究では、BPL の血漿由来製剤（例え、アルブミン、免疫グロブリン、凝固因子）のために使われた製造工程によるモデル TSE 物質スクレイピーの除去が調査された。これら製造工程の幾つかは、これらの製剤が英国血漿由来である時に使われた一方、他の製剤は米国血漿へ移行した後開発された。実験的なアプローチは、スクレイピー脳抽出物を用いて小規模プロセスモデルの個々の生産工程にスパイクし、生産工程の前後に存在するプロテアーゼ K-耐性プリオンたん白質（PrP^{SC}）の量を測定する。得られたデータは、疾病が最も高かった 1990 年代の英国血漿由来製品から vCJD リスクの評価の一助となる。

材料と方法

（略）

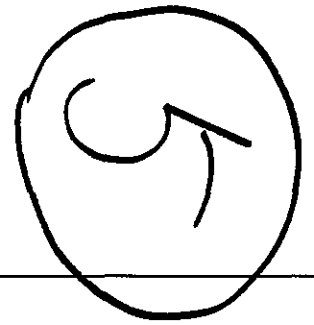
考　　察

ヒトにおける vCJD の発生の後、細胞（性）血液製剤による伝播可能な症例が今までのところ 4 つあった。しかし、血漿分画製剤の場合、このルートによる vCJD 伝播のいかなる証拠も明らかになっていない。しかし、この疾患の不顕性症例は、死亡後の患者の脾臓中に PrP^{SC} を発見した後に確認された。この症例では PrP^{SC} は低量で、そして再試験の後のみで検出された。vCJD 伝播のこの可能症例は、精製第Ⅷ因子中間体 8Y の処理によるものかもしれないと提案した一方、患者は血液成分と侵襲的手順を受けたことを含む他の関連した危険因子を持った。英国人における vCJD の既存の有病率予想は、PrP^{SC} のために多数の虫垂または扁桃腺組織の検査に基づく。陽性或いは可能性がある陽性例が分かったそれらの症例では、計算した有病率予想は、1/9,160 或いは 3/12,674 であった。しかし、他の研究では、0/32,661 の有病率が得られ、症例は見つからなかった。

伝達性海綿状脳症物質は、血漿分画製剤により潜在的に伝播するウイルスを不活化するために、一般的に用いられる物理化学的両方法に対して高い抵抗力があった。このように、存在するかもしれないいかなる TSE 物質の除去は、製造工程中に存在する標準的な区分けされた工程に頼らなければならない。幸いにも、異常プリオン・タンパク質 PrP^{SC} に関連した特徴的生理化学的性質（例えば、難溶解性、

使用上の注意記載状況・
その他参考事項等

2. 重要な基本的注意
(1)略
1)略
2)現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的な vCJD 等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。



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凝集傾向及び表面への結合）は、これらの区分けされた工程により除去が可能になりそうだ。事実、これは血漿分画製剤で幾つかの実験的研究における事例であることが示された。
現在の研究において、BPL で製造された血漿分画製剤の範囲の製造の間の代表的な TSE 物質の除去は、スクレイピーの 263K 株を用いた PrP^{SC} のウエスタンブロットによって調査された。TSE 物質は比較的小さいサイズのようで、TSE クリアランスに関する最悪の症例を代表して、広範囲に超音波処理したミクロソーム分画が確認に用いられた。スパイクの中の TSE 物質の形/サイズは、例えば、界面活性剤処理の後、理論的にもウイルス除去ができた。しかし、溶解/界面活性剤の包含と Sarkasyl 処理したスパイクの使用は、第IX因子のろ過に関する PrP^{SC} 除去に影響しなかった。類似の知見が他の血漿分画製剤のために報告された。他の調査は、ウエスタンブロットに基づくクリアランス研究の結果とバイオアッセイで測定されたそれらの間に良好な相関関係があることを示した。それにも拘わらず、これはモデル系であり、結果が他の TSE 物質と vCJD にあてはまると仮定することができるだけである。動物基準の感染性分析法が製造工程による TSE 物質の除去を評価するための至適基準である間、ウエスタンブロットによる PrP^{SC} の除去に基づく最初の評価が役立つことが証明された。現在の研究では、各々の製剤中の PrP^{SC} を取り除くための各々の生産工程によってなされる寄与が測定された。各々の製剤の結果を要約するために、各々の生産工程からの結果が互いに加えられた。しかし、この付加的なアプローチが一般的に用いられる一方、それが真の総除去能力の過大評価につながる場合があるかもしれない証拠がある。この結果は、以降の工程による除去に対してより抵抗する生産工程から残っているスパイク材料によるかもしれない。単独の最初のスパイクの後のシケンスでの全工程検査を検査することによって完全な製造工程による除去が本当に決定されることができるだけであるが、十分に高力価の TSE スパイク材料の入手が可能である場合、このスパイク材料の相違がある。
アルブミン工程の場合、(上清が保持されている) 低温沈殿反応工程は、スクレイピー物質を除去する十分な能力がないことが示された。この工程の TSE 除去能力が他の工程で分かった結果と同じ、通常約 1Log でしかなかった。セライトの様なろ過助剤による処理の効果は、以前は報告されていなかった。現在の研究において、この手順は約 1.5Log のスクレイピー物質を除去する優位な能力があった。分画 A+1 と IV 沈殿物の 2 つのエタノール分画工程は、それぞれ約 2-5Log の除去能力があった。これらの工程は、以前約 2-5Log の除去能力を持つことが示された。
デプスフィルター工程は、この手順のための 2-3Log の以前の知見と一致して、3Log の除去能力があった。イオン交換クロマトグラフィ工程から生じる除去に相当し、約 2-4Log の他の研究報告と類似していた。デプスフィルターが TSE 物質を除去することが以前にも注目された。しかし、関連した中間体の性質同様、フィルターの形とグレードは、この工程がどれくらい効果的でありえるかについて影響を与えることができる。それは、そのようなフィルターが TSE 物質を除去するために完全に効果的ではなさそうで、タンパク質のより幅広い範囲が存在する製造の初期工程が仮定された。我々の場合、用いたアルブミン中間体は比較的精製された（>95%）、そしてスクレイピー物質は約 3Log 除去された。最終滅菌ろ過工程（0.2μm）も、スクレイピー物質除去の若干の能力を示した。これは、凝集形の中に存在しているプリオンたん白質の割合によりそうである。
免疫グロブリンの場合、デプスろ過工程で除去されるスクレイピーは、アルブミンと比較すると、より制限された。Gammaplex の場合、ウイルスろ過工程（20nm）はスクレイピー物質を除去するために大変効果的であるとわかった。特に利用できる最小孔径サイズが使用されるウイルス保持フィルターは、免疫グロブリンを含む様々な血漿分画製剤から TSE 物質を除去するために効果的であるとわかった。Replenine-VF の第IX因子工程では、イオン交換吸着工程及び銅キレート・アフィニティークロマトグラフィ工程は、スクレイピー物質を除去するために効果的であることも示された。この種のアフィニティークロマトグラフィ工程が以前検査されなかった間、他のアフィニティー法、例えば、免疫親和性は TSE 物質を除去するために効果的であることが示された。銅カラムによるスクレイピー物質の除去は、銅イオンのためにプリオンたん白質の既知の親和性と一致している。しかし、第IX因子自体も銅と結合するため、クロマトグラフィー状況下でこれらの 2 つの成分のために運転中の異なる結合機構が使われなければならない。予想されるように、15nm のウイルスろ過工程の包含はこの製剤のスクレイピー除去能力を更に増やした。
精製第Ⅷ因子中間体 8Y 工程では、ヘパリン沈殿工程は TSE 除去に貢献した。クロマトグラフィ工程として、ヘパリン処理も TSE 除去に効果的であることも示された。しかし、以前に観察されたように、グリシン沈殿工程は除去に相対的に殆ど貢献していなかった。G-25

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セファデックス・クロマトグラフィ粒径排除カラム工程は、予想通り、スクレイピー物質の除去に殆どつながらない。沈殿物が保持されるグリシン/生食沈殿工程は、TSE除去に貢献しなかった。しかし、例えTSE物質が一般に沈殿分画中で見つかるとしても、この工程がTSEの約1-2Logを除去することができると報告した。全体として、最高の工程で1.8Log貢献し、追加により推定される合計TSE除去は、合計3.3Logで比較的制限された。より高純度第Ⅷ因子製剤Optivateは、更に4.1LogまでTSE除去を実質的に増やす追加の陽イオン交換クロマトグラフィ工程を含んだ。以前の研究は、第Ⅷ因子製剤の製品間のTSE物質の2-4Logを除去することができるDEAEベースの陰イオン交換クロマトグラフィ工程を示した。
現在の研究は、かなりのTSE除去が製品検査の異なるタイプの各々で得られたことを証明した。多様な生産工程が製造工程に関係しているとき、TSE除去は一般により大きかった。しかし、幾つかの工程、例えばエタノール分画、デプスろ過、アフィニティ或いはイオン交換クロマトグラフィは、特に効果的だった。ウイルスろ過工程が含まれたより新しい製剤については、主にウイルス安全性を増やす目的で、この工程もTSE物質を除去するために特に効果的であることが示された。この種のアプローチは、通常、多くの血漿分画製剤に適用できる。しかし、非常に高いTSE量が含まれるとき、これらのフィルターでさえ除去のために約3Logの有効能力を恐らく持っているだろう。今回と以前の研究は、血漿分画製剤からTSE物質を除去するために、通常、多くの製造工程の効果を示した。しかし、血液中のプリオンの正確な性質がまだ完全には理解されていないため、そのようなスパイク研究を説明するとき、注意が必要である。少しの感染性だけが実験動物TSEモデルにおける血液と関係していることが分かっているが、これのかなりの割合は血漿成分自体と関係している。このように、低い潜在的TSE量は、TSE物質を除去する製造工程の能力と共に、血漿分画製剤によるvCJD伝播の見込みを大幅に減らさなければならない。
8Y工程の様な精製第Ⅷ因子中間体のTSE除去能力は、以前はわずか約1Logと推定されていた。しかし、現在の研究に基づいて、これは過小評価を意味するかもしれない、そして、最も効果的な工程の2Logと追加による全工程の3Logで、完全な8Y製造工程の除去データが考慮されたとき、より大きな除去はより適切になるだろう。英国血漿が過去に使われた所でさえ、これはこの製剤の安全性に対する有意な貢献をすることができた。事実、そのような原料に関連した感染性を暗示した英国血漿由来の凝固因子を投与された人々のvCJDの臨床症例の欠如は、過大評価されたかもしれない。
いろいろな血漿分画製剤の製造工程での固有のTSE除去能力に加えて、米国血漿由来製剤への変更は、危険性において更に相当な除去をもたらした。米国では、FDAは米国製第Ⅷ因子からのvCJD感染の危険は一般的に非常に低くなりそうだが、ゼロではないと結論した。第Ⅷ因子8Y工程のTSE除去能力は検査した他の血漿分画製剤のそれより低かったが、この製剤が本来英国血漿から製造された時代に、この物質の伝播を不可能にするのに十分である場合があった。その上、BSE或いはvCJDが起こっていない地方から集めた血漿の使用は、これらの製剤の改善された安全性に貢献する主要な要因でもある。

報告企業の意見	今後の対応
血漿分画製剤は理論的なvCJD伝播リスクを完全には排除できないため、投与の際には患者への説明が必要である旨を2003年5月から添付文書に記載している。2009年2月17日、英国健康保護庁(HPA)はvCJDに感染した供血者の血漿が含まれる原料から製造された第Ⅷ因子製剤の投与経験のある血友病患者一名から、vCJD異常プリオン蛋白が検出されたと発表したが、日本血液製剤機構の原料血漿採取国である日本では、欧州滞在歴のある献（供）血希望者を一定の基準で除外し、また国内でのBSEの発生数も少数であるため、原料血漿中に異常型プリオン蛋白が混入するリスクは1999年以前の英国に比べて極めて低いと考える。また、本剤の製造工程においてプリオンが低減される可能性を検討するための実験を継続して進めているところである。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

JBPO 2012-004

VoxSanguinis

The International Journal of Transfusion Medicine

ISBT International Society of Blood Transfusion

Vox Sanguinis (2012)

DOI: 10.1111/vox.12004

ORIGINAL PAPER

Removal of TSE agent from plasma products manufactured in the United Kingdom

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Vox Sanguinis

Background and Objectives The outbreak of vCJD in the UK leads to concern regarding the potential for human-to-human transmission of this agent. Plasma-derived products such as albumin, immunoglobulin and coagulation factors were manufactured by BPL from UK plasma up until 1999 when a switch to US plasma was made. In the current study, the capacity of various manufacturing processes that were in use both prior to and after this time to remove the TSE agent was tested.

Materials and Methods Small-scale models of the various product manufacturing steps were developed. Intermediates were spiked with scrapie brain extract and then further processed. Samples were assayed for the abnormal form of prion protein (PrP^{SC}) by Western blotting, and the reduction in the amount of scrapie agent determined.

Results Many of the manufacturing process steps produced significant reduction in the scrapie agent. Particularly effective were steps such as ethanol fractionation, depth filtration, ion-exchange and copper chelate affinity chromatography. Virus retentive filters, of nominal pore size 15 or 20 nm, removed >3 log. The total cumulative reduction capacity for individual products was estimated to range from 7 to 14 log. In the case of factor VIII (8Y), the total removal was limited to 3 log.

Conclusion All the processes showed a substantial capacity to remove the TSE agent. However, this was more limited for the intermediate purity factor VIII 8Y which included fewer manufacturing steps.

Received: 3 April 2012,
revised 1 October 2012,
accepted 2 October 2012

Key words: biosafety, manufacturing steps, plasma products, TSE agent removal, vCJD.

Introduction

Transmissible spongiform encephalopathies (TSE) are rare neurodegenerative diseases of humans and other animals (1, 2). The emergence of bovine spongiform encephalopathy (BSE) in the UK in 1986 gave cause for concern in view of the possibility of transmission to humans. This proved to be a reality when the first cases of the human form of this disease, known as variant Creutzfeldt-Jakob disease (vCJD) was recognised in 1995 (3). The establishment of this disease in humans raised the possibility that human-to-human transmission, from asymptomatic carriers, could potentially occur via blood components or plasma products (4). Concerns over this potential risk for plasma products derived from large numbers of donors led the Department of Health for England to switch in 1999 from UK plasma to US plasma for fractionation by the Bio Products Laboratory Ltd (BPL, Elstree, UK). This was seen as an immediate safety measure to reduce the theoretical transmission risks given that vCJD appears to be essentially absent in the US apart from cases that have arisen outside the country.

In the case of plasma products, there has been no clear evidence for the transmission of vCJD disease (5). However, there have been four likely cases of transmission by cellular blood products (6). In addition, a subclinical case of vCJD infection has also been identified by finding PrP^{SC} in a patient's spleen at post-mortem (7, 8); the patient had

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received intermediate purity factor VIII (8Y) manufactured from plasma derived from UK plasma amongst other risk factors.

While there is data available for some plasma products to show that the manufacturing processes are generally able to remove TSE agents (9, 10), there are no data available for the products manufactured by BPL. In the current study, the removal of the model TSE agent scrapie by the manufacturing processes used for BPL's plasma-derived products (i.e. albumin, immunoglobulin and coagulation factors) has been investigated. Some of these manufacturing processes were in use when these products were originally derived from UK plasma, whilst others were developed after the transfer to US plasma. The experimental approach used involved spiking individual process steps of small-scale process models with scrapie brain extract and determining the amount of protease K-resistant prion protein (PrP^{SC}) present before and after the process step. The data obtained can help to assess the vCJD risk from UK plasma-derived products during the 1990s when the epidemic was at its highest.

Materials and methods

Spike material

The 263K hamster adapted strain of the scrapie agent was used. A brain extract was prepared from inoculated animals in the late phase of the disease by homogenisation in phosphate buffer saline (PBS). At this stage, this material typically had an infectivity titre of about 10^7–10^8 ic LD_{50} units/ml in hamsters. From this, a microsomal extract was prepared based on the method described by Millson *et al.* (11). This procedure has been shown to produce a preparation containing essentially fragmented endoplasmic reticulum and ribosomes. This was carried out by Dounce homogenisation of infected brain tissue in PBS followed by centrifugation at 10 000 *g* for 7 min to remove cells, organelles and large aggregates. This was then further centrifuged at 100 000 *g* for 1 h, and the pellet resuspended in PBS and stored at −70°C.

This type of spike was chosen as a compromise between a simple crude brain homogenate that contains significant amounts of high molecular weight host material and extensively purified scrapie agent which can tend to aggregate. To minimise the size of the scrapie agent fragments present in the preparation, the material was extensively sonicated, based on the method described by Yunoki *et al.* (12), to give a particle size of about 100 nm. This was carried out immediately before use using a probe sonicator (Bio 70; Ultrasonic Engineering, Middlesex, UK) with settings of Operation 5, Sonics 3 and Amplitude 100. Treatment was carried out for 5 min on ice.

Process intermediates

Process intermediates for each of the relevant plasma products (Fig. 1) were provided by the production department at BPL. The BPL products evaluated were as follows: Albumin (Zenalb® 4·5, 20) (13), Subcutaneous (Subgam®) and Intravenous Immunoglobulin (Vigam®-S & Liquid, Gammaplex®) (14, 15), Factor VIII/Von Willebrand Factor (VWF) (Dried factor VIII fraction type 8Y, Optivate®) (16, 17) and Factor IX (Replenine®, Replenine®-VF) (18).

Process models

Small-scale models of the manufacturing process (Fig. 1, Table 2–5) were used for evaluating reduction in the scrapie agent. The models were a direct physical scale-down of the full-scale process. Prior to the scrapie agent spiking studies, runs were carried out with product intermediate alone to ensure that the appropriate process parameters such as product yield and activity were within those defined for the full-scale manufacturing process. In all cases, the intermediates used were obtained from the BPL production department, before spiking with scrapie brain extract and subsequent processing. Samples were taken after spiking and at the end of the process step and stored at −70°C before assay.

Albumin

Cryoprecipitation was carried out by spiking pooled plasma (133 ml) with 7 ml of scrapie agent. This was frozen at −70°C as a thin layer and then stored at −10 to −20°C. The material was then crushed and transferred to a prechilled reaction vessel at 0–2°C and stirred for 30 min. The cryoprecipitate was collected by centrifugation at 5600 *g* for 4 min in a swing-out rotor at 3°C. The supernatant was then poured off, and the precipitate suspended in 20 ml of PBS.

For the Celite treatment step, 6 g of Celite was added to 1100 g of cryosupernatant (CPS), mixed for 150 min at 3°C and then clarified through a Seitz depth filter (K250C). This material was used to precondition a new Seitz depth filter.

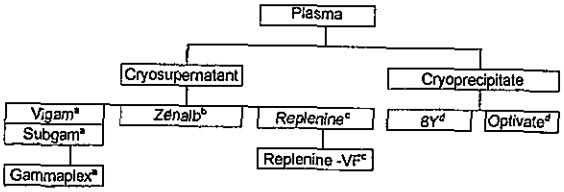

Fig. 1 Flow diagram for the manufacture of plasma products: [a]immunoglobulins, [b]albumin, [c]factor IX, [d]factor VIII. Further details are given in Tables 1–4.

This was followed by filtration of a further 103 g of cryosupernatant spiked with 11 g of scrapie agent and treated with 0·6 g Celite at 1·5 bar. Ethanol fractionation was then carried out by the Kistler–Nitschmann process (13, 19). The A + 1 precipitation step was carried out by spiking 133 ml of Celite treated intermediate with 7 ml of scrapie agent. This was then transferred to a prechilled reaction vessel, mixed, and cold ethanol added over an 8 h period to reach a final concentration of 19% (v/v) at −5°C. The mixture was allowed to mature over a 6 h period before being collected by centrifugation at 5600 *g* in a swing-out rotor for 9 min at −5°C. The A + 1 supernatant was decanted, and the pellet resuspended in 20 ml of PBS.

Fraction IV precipitation was carried out by taking 133 ml of A + 1 supernatant and spiking with 7 ml of scrapie agent. This was added to a prechilled reaction vessel, mixed, and ethanol added over a 10 h period to give a final concentration of 40% (v/v) at −5°C. After maturation for 6 h, the material was centrifuged at 5600 *g* for 16 min at −5°C in a swing-out rotor, and the supernatant retained. This was assayed directly, that is, without including a further depth filtration step. For the fraction V depth filtration step, a Cuno Zeta Plus BioCap 90LPZ depth filter (Beckman Coulter, High Wycombe, UK) was conditioned with 210 g of fraction V solution at pH 7·4. This was followed by filtering 98 g of fraction V spiked with 11 g of scrapie agent at 9°C and 1·5 bar, and the filtrate collected.

The final polishing step was carried out by chromatography using DEAE-Sepharose fast flow (GE Healthcare Life Sciences, Little Chalfont, UK) on a 25 ml column (12 cm height) at 7°C. After equilibration with buffer at pH 4·6, 300 ml of redissolved and diafiltered fraction V precipitate, spiked with 10 ml of scrapie agent, was applied. The flow-through albumin containing fraction was retained.

The final sterile filtration step was evaluated by spiking 99 g of final formulated 20% albumin with 11 g of scrapie agent. This was then filtered through a 0·2 μm Pall Ultipore or Millipore Durapore polyvinylidine floride filter (9 cm^2) at room temperature and a pressure of 1·8 bar.

Immunoglobulins

The manufacturing process for the intravenous immunoglobulins Vigam and Gammaplex and the subcutaneous immunoglobulin Subgam was essentially similar except for the addition of a terminal virus filtration step in the case of Gammaplex. The cryoprecipitation, Celite treatment and A + 1 precipitation steps were as described for albumin.

For the B + 1 precipitation step (13, 19), 133 ml of redissolved A + 1 precipitate was spiked with 7 ml of scrapie agent and transferred to a chilled reaction vessel and stirred. Ethanol was slowly added, with stirring, over an 8 h period to give final conditions of 17% (v/v) pH 5·1 and −3°C. This was then left for a further 7 h before centrifugation at 5600 *g* in a swing-out rotor for 7 min at −5°C. The precipitate was then resuspended in 20 ml of PBS. A further depth filtration step was not included.

The fraction II depth filtration step was tested by conditioning a Cuno Zeta Plus BioCap 60LPZ depth filter with 400 g of redissolved fraction II paste pH 6·6. This was then followed by 97 g of intermediate spiked with 11 g of scrapie agent at 10°C and 1 bar, and the filtrate collected.

The DEAE-Sephadex adsorption step was carried out using 98 g of fraction II solution at pH 6·7, which was spiked with 5 ml of scrapie agent at 8°C. DEAE-Sephadex was then added (3 g/g protein) and mixed for 1 h before the gel was removed under vacuum, using a sintered funnel, and the filtrate collected.

For the CM-Sepharose chromatography step, 80 g of solvent/detergent-treated and soybean oil extracted intermediate at pH 4·0 was spiked with 4 ml of scrapie agent at 10°C. After loading and washing the column, the IgG was eluted using 0·17 M sodium chloride, 0·02 M sodium acetate and 0·08 M glycine at pH 9·0, and the peak fraction collected.

Virus filtration was carried out using 118 ml of CM-Sepharose eluate spiked with 2 ml of scrapie agent. This was prefiltered through a Pall DVD filter before virus filtration through a 0·001 m^2 Pall DV20 filter at 2–3 bar at room temperature.

Factor IX

The cryoprecipitation and DEAE-Sepharose steps were as described for albumin and immunoglobulins. For the copper-charged chelating Sepharose chromatographic step, 110 ml of solvent/detergent-treated intermediate was spiked with 7 ml of scrapie agent. This was then loaded on to a column of 31 ml bed volume (16 cm bed height) and washed sequentially with citrate buffers containing 100–500 mM sodium chloride at pH 6·5, pH 7·0, pH 4·4 and pH 7·0. Finally, the factor IX was eluted with 10 mM sodium citrate, 1·4 M citric acid, 10 mM sodium phosphate, 100 mM sodium chloride and 20 mM glycine pH 7·0, and the peak fraction collected.

In the case of Replenine-VF, an additional virus filtration step was included in the process. Copper chelate column eluate (66 ml) was spiked with 4 ml of scrapie agent that had been treated with detergent (1% Sarkasyl). In some experiments, solvent/detergent (1% polysorbate 80 and 0·3% tri-*N*-butyl phosphate) was also added to the Replenine intermediate. Virus filtration was carried out using an Asahi Planova 15 N filter of 0·001 m^2 at 1 bar. This was followed by 28 ml of 10 mM sodium citrate, 10 mM sodium phosphate, 100 mM sodium chloride and 20 mM glycine pH 7·0 wash.

Factor VIII

The cryoprecipitation step was as described for albumin. The heparin precipitation was tested by spiking 175 ml of cryosupernatant spiked with 9 ml of scrapie agent at 25°C. Heparin was then slowly added to reach a final concentration of 88 mg/100 g. After mixing for 2 min, the solution was centrifuged at 5000 *g* for 10 min in a swing-out rotor, and the supernatant retained. A sample of the heparin supernatant was then mixed at 25°C and glycine/saline buffer pH 7·0 added. This was further mixed for 5 min, centrifuged at 5600 *g* for 30 min in a swing-out rotor, and the precipitate redissolved in tris/citrate buffer at pH 6·9 and 30°C.

The Sephadex G-25 column step was tested using a 100 ml column (50 cm bed height). This was loaded with 18 g of redissolved heparin precipitate spiked with 1 ml of scrapie agent and eluted with tris/citrate buffer pH 6·9 containing 0·1 M sodium chloride and 1·5% of sucrose, and the peak fraction collected.

In the case of Optivate, 80 ml of solvent/detergent (1% polysorbate 20 and 0·3% tri-*N*-butyl phosphate)-treated redissolved glycine saline precipitate was spiked with 4 ml of scrapie agent. This was then loaded onto an 80 ml column (15 cm bed height) cation-exchange column. This was then washed with low salt tris/citrate buffer pH 6·5, before elution with the same buffer containing 1·1 M sodium chloride, 5 mM calcium chloride and 0·1% polysorbate 20.

Spiking studies

Process intermediates were spiked with scrapie agent as described above. The relevant process model was then performed. Samples taken before and after the step were then assayed for PrP^{SC} by Western blotting as described below. Samples to be compared were run on the same gel to minimise any between-assay variation and thus ensure the best comparison. The PrP^{SC} titres were expressed as a reciprocal of the dilution end-point and, after correction for the total volume assayed and the volume of the intermediate, used to determine the total PrP^{SC} titre. The log reduction value was calculated by subtracting the total log PrP^{SC} remaining after the treatment step from that present at the start. All reduction values were considered significant unless relatively small, that is, below three-fold/0·5 log.

Western blot assay

The PrP^{SC} titre was determined by a Western blot method based on that described previously (20, 21). This method relies on the difference in susceptibility of the resistant infectious (PrP^{SC}) and normal (PrP^{C}) forms of the prion protein to protease digestion. In some experiments, the samples were prepared by treatment with sodium phosphotungstate to precipitate the PrP^{SC}. This process was also used to reduce the level of the host cell background in the Western blotting assay and to concentrate the PrP^{SC} present in the sample. The samples were adjusted to neutral pH and digested with an optimised concentration of Protease K to remove any normal PrP^{C} that might be present. A three-fold dilution series of each sample were then prepared, denatured and subjected to polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, washed, and the protein bands detected using the PrP-specific monoclonal antibody 3F4 (22) followed by alkaline phosphatase conjugated secondary antibody. The PrP^{SC}-specific bands were identified by reference to the bands present in a control scrapie brain extract sample. The end-point was taken as the last dilution where a PrP^{SC} band could be detected in the test sample but was absent in the control unspiked sample. The validity of this approach for quantifing PrP^{SC} in process samples was tested in initial studies as outlined in Fig. 2.

Results

Western blot assay for PrP^{SC}

The suitability of the Western blot method for evaluating the capacity of the various manufacturing process steps to remove PrP^{SC} was initially tested using samples taken from

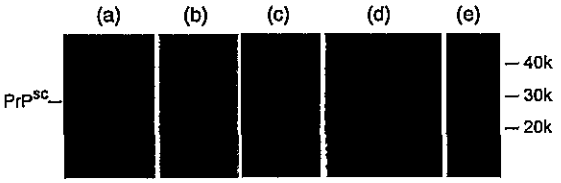

Fig. 2 Evaluation of assay for PrP^{SC} removal by Western blotting in process samples from the Heparin Sepharose chromatography step of the Factor XI process. Intermediate was spiked 1:50 with scrapie, and Heparin Sepharose added at 4°C and mixed. The gel was then transferred to a chromatography column and washed with buffer at pH 7. The product was then eluted using phosphate buffer at pH 6·4 containing 2 M sodium chloride. Samples were digested with protease K, denatured, run on a polyacrylamide gel and transferred to a PVDF membrane. After washing, PrP^{SC} bands were detected using labelled 3F4 antibody. (a) Microsomal extract of scrapie infected hamster brain, three-fold dilution series. (b) Nonspiked process intermediate showing column load, wash and product eluate, respectively. (c) Spiked column load, three-fold dilution series. (d) Spiked column flow-through, three-fold dilution series. (e) Spiked column eluate, three-fold dilution series. The position of the main protease resistant prion protein band (PrP^{SC}) is indicated, together with molecular weight markers of 40, 30 and 20 kD.

the chromatographic manufacturing process for factor XI as an example (Fig. 1). The protease K-treated samples, including a microsomal scrapie brain extract and spiked process samples, showed a series of bands in the molecular weight range of about 20–30 K as expected for the PrP^{SC} protein. The three-fold dilution series showed that the intensity of the major band decreased proportionally with sample dilution. This thus allowed the quantitation of PrP^{SC} by end-point dilution and enabled a titre to be calculated. While a PrP^{SC} reactive band was apparently detectable in all undiluted samples of the unspiked column load, flow-through and product eluate, this did not co-migrate with the PrP^{SC} bands themselves. The PrP^{SC} protein could be detected and quantified in the column load and flow-through fraction. However, the PrP^{SC} band was absent from the product eluate fraction. When fraction volume is taken into account with the PrP^{SC} titre/ml, the total PrP^{SC}/fraction could be determined. From this, the total removal by the process could be calculated. In this case, the Heparin Sepharose process removed about 2·7 log of scrapie agent PrP^{SC}.

Albumin

The removal of scrapie agent PrP^{SC} during the various manufacturing steps of the Zenalb process is shown in Table 1. Several steps had some (2 log) capacity to remove the scrapie agent. However, depth filtration of fraction V and ion-exchange chromatography were particularly effective (3–4 log). Because TSE agents have been shown to mainly partition into precipitate fractions (9, 10), the fraction V precipitation step, in which the precipitate is retained, was not tested. Given the relatively mild conditions used for the terminal pasteurisation, namely, 60°C for 10 h, this step was considered unlikely to cause the direct inactivation of TSE agents by denaturing the PrP^{SC} protein. In addition, this step will have no capacity for TSE removal and thus was not tested. The log reduction obtained for the most efficient step in the manufacturing process was 3·5 log. The reduction value for the total process, assuming that all the steps are additive, was 13·6 log.

Table 1 The removal of scrapie agent from Albumin: Zenalb®

	Total PrP^{SC} (log)		
Process Step[a]	Initial	Final	Removal
Cryoprecipitate Supernatant	7·6	7·6	0·0
Celite Treatment	5·6	4·1	1·5
Fraction A + 1 Supernatant	7·3	4·8	2·5
Fraction IV Supernatant	7·8	5·4	2·4
Fraction V Precipitate	ND	ND	ND
Fraction V Depth Filtration	6·2	3·4	2·8
Ion-Exchange Chromatography	6·6	3·1	3·5
Sterile Filtration	4·8	3·9	0·9
Pasteurisation	ND	ND	ND
Total[b]			13·6

[a]Originally manufactured from UK plasma and later from US plasma.
[b]Assuming all steps are additive.
ND, Not determined.

Immunoglobulins

For intravenous (Vigam and Gammaplex) and subcutaneous (Subgam) immunoglobulins, there were four steps in the basic process with some capacity (1–2 log) to remove the scrapie agent PrP^{SC} (Table 2). The fraction II precipitation was not tested as again this step was thought unlikely to contribute to the removal of the TSE agent as the precipitate is collected in this case. The log reduction obtained for the most efficient step was 1·9 log and for the total process, as estimated by the addition of all the process steps, was about 6·8 log. The solvent/detergent and terminal low pH incubation steps were not tested in view of the known high resistance of the scrapie agent to inactivation/denaturation and the absence of any capacity for removal by these procedures. In a further development of this product, Gammaplex, a 20 nm virus filtration step was introduced, and this was able to remove >2·6 log of the scrapie agent, with that estimated for the entire process being >9·4 log.

Table 2 Removal of scrapie agent from Immunoglobulins: Vigam®, Subgam® and Gammaplex®

	PrP^{SC} Removal (log)	
Process Step	Vigam[a], Subgam[b]	Gammaplex[b]
Cryoprecipitate Supernatant	0·0	0·0
Celite Treatment	1·5	1·5
Fraction A + 1 Precipitate	0	0
Fraction B + 1 Supernatant	1·0	1·0
Fraction II Precipitate	ND	ND
Fraction II Depth Filtration	0·6	0·6
DEAE-Sephadex Adsorption	1·8	1·8
Solvent/Detergent	ND	ND
CM-Sepharose Chromatography	1·9	1·9
Virus Filtration	NA	>2·6
Terminal Low pH Incubation	ND	ND
Total[c]	6·8	>9·4

[a]The freeze-dried form of this product (Vigam -S) was originally manufactured from UK plasma and the later liquid form (Vigam liquid) from US plasma.
[b]Manufactured from US plasma.
[c]Assuming all steps are additive.
ND, Not determined; NA, Not applicable.

Factor IX

The Replenine manufacturing process contained two significant steps of 2 to >3 log for removing the scrapie agent (Table 3). The copper chelate affinity chromatography step was particularly effective. Of the total PrP^{SC} that was applied to the column, about 20% was detected in the flow-through, and the remainder was most likely to have bound to the column and been removed during the column recycling process. The factor IX process was later modified to include an additional 15 nm virus filtration step, and this was also tested. In this case, the scrapie brain extract spike was used either in the absence or presence of solvent/detergent as a potential theoretical worst case. PrP^{SC} removal by the filter was similar in both cases, with a mean of >2·9 log. The log reduction obtained for the most efficient step in the process was >3·4 log with the estimate for the entire process being >8·1 log.

Factor VIII

Given that the heat treatment step was only carried out at 80°C, this step was considered unlikely to cause the direct inactivation of TSE agents by denaturing the PrP^{SC} protein. In addition, this step will have no capacity for TSE removal and thus was not tested for either of the products. The intermediate purity factor VIII product 8Y contained two steps each with a scrapie agent PrP^{SC} removal capacity of about 1·5–2 log (Table 4). The reduction for the most effective step in the 8Y process was 1·8 log with the total for the complete process estimated as up to about 3·3 log. In the case of the factor VIII/VWF concentrate Optivate, the addition of a cation-exchange chromatography step provided an extra 4 log of PrP^{SC} removal, giving a total of up to 7·4 log for the complete process.

Table 3 Removal of scrapie agent from factor IX: Replenine® and Replenine®-VF

	PrP^{SC} Removal (log)	
Process Step	Replenine[a]	Replenine-VF[b]
Cryoprecipitate Supernatant	0·0	0·0
DEAE-Sepharose Adsorption	1·8[d]	1·8[d]
Solvent/Detergent	ND	ND
Copper Chelate Chromatography	>3·4	>3·4
Virus Filtration	NA	>2·9
Total[c]	>5·2	>8·1

[a]Manufactured from UK plasma.
[b]Manufactured from US plasma.
[c]Assuming all steps are additive.
[d]Value estimated from the DEAE step used in the IgG process (Table 2).
ND, Not determined; NA, Not applicable.

Table 4 Removal of scrapie agent from factor VIII: 8Y® and Optivate®

	PrP^{SC} Removal (log)	
Process Step	8Y[a]	Optivate[b]
Cryoprecipitate	1·5	1·5
Heparin Supernatant	1·8	1·8
Glycine/Saline Precipitate	0·1[c]	0·1[c]
Sephadex G-25 Chromatography	<0·3[c]	NA
Solvent/Detergent	NA	ND
Ion-Exchange Chromatography	NA	4·1
Dry-Heat Treatment	ND	ND
Total	3·3	7·4

[a]Originally manufactured from UK plasma and currently from US plasma.
[b]Manufactured from US plasma.
[c]Excluded from total.
ND, Not determined; NA, Not applicable.

Discussion

Following the emergence of vCJD in the human population, there have been up to four possible cases of transmission by cellular blood products (6). However, in the case of plasma products, there has been no clear evidence for any vCJD transmission by this route (5). However, a possible subclinical case of this disease has since been identified after finding PrP^{SC} in a patient's spleen at post-mortem. In this case, PrP^{SC} was detected at a low level and only after repeated testing (7). Whilst it has been proposed that this possible case of vCJD transmission may have been due to treatment with the intermediate purity factor VIII 8Y, the patient had other associated risk factors including having received blood components and invasive procedures (8). The existing prevalence estimates for vCJD in the UK population are based on testing large numbers of appendix or tonsil tissue for PrP^{SC}. In those cases where positive or possible positive cases have been found, the prevalence estimates calculated were one in 9160 (23) or three in 12 674 (24). However, in other studies, no cases were detected (25, 26), giving a prevalence of 0 in 32 661 (26).

Transmissible spongiform encephalopathy agents are highly resistant to both the physical and chemical methods that are commonly used for inactivating the viruses that are potentially transmitted by plasma products (27–29). Thus, the elimination of any TSE agents that may be present must rely on the standard partitioning steps present in the manufacturing process. Fortunately, the characteristic physicochemical properties associated with the abnormal prion protein PrP^{SC}, such as low solubility, tendency to aggregate and bind to surfaces, make it likely that it can be removed by these partitioning steps (30). Indeed, this has been shown to be the case in several experimental studies with plasma products (9, 31).

In the current study, the removal of a representative TSE agent during the manufacture of a range of plasma products manufactured by BPL has been investigated by Western blotting for PrP^{SC} using the 263K strain of scrapie (Table 5). An extensively sonicated microsomal fraction was used to ensure that the TSE agent was likely to be of a relatively small size and thus to represent a worst case in terms of TSE clearance (12, 32). The form/size of the TSE agent in the spike, for example, after detergent treatment, could in theory also effect virus removal (33). However, the inclusion of solvent/detergent and the use of a Sarkasyl-treated spike did not influence PrP^{SC} removal on filtration with factor IX. Similar findings have been reported for other plasma products (33). Other studies have shown that there is a good correlation between the results of clearance studies based on Western blotting and those determined by bioassays (34–36, 38). Nevertheless, this is a model system, and it can only be assumed that the results apply to other TSEs and vCJD. Whilst animal-based infectivity assays remain the gold standard for assessing the removal of TSE agents by manufacturing process, initial evaluations based on the removal of PrP^{SC} by Western blotting (37) have proved useful. In the current study, the contribution made by each process step for removing PrP^{SC} in each product has been determined. To summarise the results for each product, the results from each process step have been added together. However, while this additive approach is commonly used, there is evidence that it may in some cases lead to an overestimate of the true total removal capacity (9). This effect may be due to the spike material remaining from a process step being more resistant to removal by a subsequent step. Although the removal by a complete manufacturing process can really only be determined by testing all the steps in sequence after a single initial spike, the availability of TSE spike material of sufficiently high titre can make this difficult.

Table 5 Removal of scrapie agent from plasma products: Summary

	PrP^{SC} Removal (log)	
Product	Maximum Individual step	Total Process
Albumin		
Zenalb®	3·5	13·6
Immunoglobulin		
Vigam®, Subgam®	1·9	6·8
Gammaplex®	>2·6	>9·4
Factor IX		
Replenine®	>3·4	>5·2
Replenine®-VF	>3·4	>8·1
Factor VIII		
8Y®	1·8	3·3
Optivate®	4·1	7·4

In the case of the albumin process, the cryoprecipition step, in which the supernatant is retained, was shown to have no significant capacity to remove the scrapie agent. This is in agreement with the results found by others where the TSE removal capacity of this step has generally been no more than about 1 log (32, 38, 39). The effect of treatment with a filter aid such as Celite has not been reported previously. In the current study, this procedure had a significant capacity to remove the scrapie agent by about 1·5 log. The two ethanol fractionation steps, fraction A + 1 and IV precipitation, each had a removal capacity of about 2·5 log. These steps have been shown previously to have a reduction capacity of about 2–5 log (9, 33, 34, 39).

The depth filtration step had a reduction capacity of 3 log, in agreement with previous findings of 2–3 log for this procedure (9, 33). The reduction resulting from the ion-exchange chromatography step was substantial and similar to that reported in other studies of about 2–4 log (9, 33, 39). It has also been noted previously that depth filtration can remove TSE agents (21, 33, 39, 40). However, the type and grade of filter, as well as the nature of the intermediate involved, can influence how effective this process can be. It has been postulated that at an early stage of manufacture when a much broader range of proteins are present, such filters are less likely to be completely effective for removing TSE agents (9). In our case, the albumin intermediate used was relatively pure (>95%), and the scrapie agent was removed by about 3 log. The final sterile filtration step (0·2 μm) also showed some capacity for scrapie agent removal. This is likely to be due to a proportion of the prion protein existing in an aggregated form.

In the case of immunoglobulin, scrapie removal by the depth filtration step was, in comparison with albumin, more limited. In the case of Gammaplex, a virus filtration step (20 nm) proved very effective for removing the scrapie agent. Virus retentive filters, particularly where the smallest pore size available is employed, have proved effective for removing TSE agents from various plasma products (41) including immunoglobulin (36, 42, 43).

In the factor IX process for Replenine-VF, the ion-exchange adsorption step and the copper chelate affinity chromatography step were also shown to be effective for removing the scrapie agent. Whilst this type of affinity chromatography step has not been tested before, other affinity methods, for example, immunoaffinity, have been shown to be effective for removing TSEs (9, 44). The removal of the scrapie agent by the copper column is consistent with the known affinity of the prion protein for copper ions (45). However, as factor IX itself also binds to copper, there must be a differential binding mechanism in operation for these two components under the chromatographic conditions used. As expected, the inclusion of a

15 nm virus filtration step further increased the scrapie removal capacity for this product.

In the intermediate purity factor VIII 8Y process, the heparin precipitation step contributed to TSE removal. Heparin treatment, as a chromatography process, has also been shown to be effective for TSE removal (35). However, the glycine precipitation step, as observed previously (32), contributed relatively little to removal. The G-25 Sephadex chromatography size-exclusion column step, as expected, leads to little removal of the scrapie agent. The glycine/saline precipitation step, in which the precipitate is retained, did not contribute to TSE removal. However, it has been reported that this step may be capable of removing about 1–2 log of TSE (35) even though TSEs generally are found in the precipitate fraction. Overall, the total TSE removal estimated by addition was relatively limited at a total of 3·3 log, with the best step contributing 1·8 log. The higher purity factor VIII product Optivate contained an additional cation-exchange chromatography step which substantially increased TSE removal by a further 4·1 log. Previous studies have shown a DEAE-based anion-exchange chromatography step to be capable of removing 2–4 log of TSE agent during the manufacture of factor VIII products (33, 39).

The current study has demonstrated that substantial TSE removal was obtained with each of the different types of products tested. TSE removal was generally greater when multiple process steps are involved in the manufacturing process. However, some steps, such as ethanol fractionation, depth filtration, affinity or ion-exchange chromatography, were particularly effective. For newer products where a virus filtration step has been included, principally with a view to increasing virus safety, this step was also shown to be particularly effective for removing TSE agents. This type of approach is generally applicable to many plasma products. However, even these filters probably have a finite capacity of about 3 log for removal when very high TSE loads are involved (12, 46). This and previous studies have generally shown the effectiveness of many manufacturing process steps for removing TSE agents from plasma products (9, 10, 47). However caution is needed when interpreting such spiking studies because the exact nature of the prion in blood is still not fully understood. It has been found that only a small amount of infectivity is associated with blood in experimental animal TSE models, although a significant proportion of this is associated with the plasma component itself (48). Thus, the low potential TSE load, together with the ability of the manufacturing processes to remove TSE agents, should substantially reduce the likelihood of vCJD transmission by plasma products.

The TSE reduction capacity of the intermediate purity factor VIIIs such as the 8Y process has previously been estimated to be only about 1 log (49). However, based on the current study, this may represent an underestimate and a greater reduction may be more appropriate when removal data for the full 8Y manufacturing process is taken into account, with 2 log for the most effective step and 3 log for the total process by addition. This could make a significant contribution to the safety of this product, even where UK plasma has been used in the past. Indeed, the absence of clinical cases of vCJD in those that receive clotting factors derived from UK plasma has led to the suggestion that the infectivity associated with such material may have been overestimated (50).

In addition to the TSE reduction capacity inherent in the various plasma product manufacturing processes, the change to products derived from US plasma will have made a further substantial reduction in risk. In the US, the FDA have concluded that the risk of vCJD infection from US-manufactured factor VIII generally appears likely to be very low but may not be zero (51). Although the TSE removal capacity of the factor VIII 8Y process was lower than that for the other plasma products tested, it may have been sufficient to make the transmission of this agent, at the time when this product was originally manufactured from UK plasma, unlikely. In addition, the use of plasma collected from regions where BSE or vCJD are absent is also a major factor contributing to the improved safety of these products.

Acknowledgements

We would like to thank Sarah Kingsland, John More, Tara Dolan, Philip Marshall, Chris Hardway for advice on products and processes and also staff at BioReliance for carrying out some of the Western blot assays.

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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	新医薬品等の区分	総合機構処理欄
				2012. 9. 19	該当なし	

一般的名称	解凍人赤血球濃厚液	研究報告の公表状況		公表国
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社) 解凍赤血球液-LR「日赤」(日本赤十字社) 照射解凍赤血球液-LR「日赤」(日本赤十字社)		McMullan LK, Folk SM, Kelly AJ, MacNeil A, Goldsmith CS, Metcalfe MG, Batten BC, Albariño CG, Zaki SR, Rollin PE, Nicholson WL, Nichol ST. N Engl J Med. 2012 Aug 30;367(9):834-41.	米国

研究報告の概要

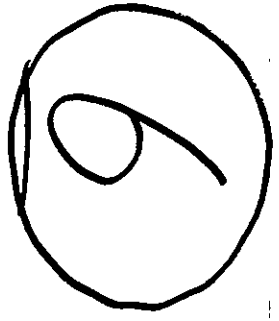
○ミズーリ州における重症熱性疾患と関連する新規フレボウイルス
ミズーリ州北西部の二人の男性が、発熱、疲労、下痢、血小板減少及び白血球減少で別々に医療機関を受診した。二人は発症5～7日前にダニに刺されていた。*Ehrlichia chaffeensis*が原因であると疑われたが、血清学的分析、PCRアッセイまたは細胞培養により陰性であった。電子顕微鏡検査でブニヤウイルス科のウイルスの存在が明らかになった。次世代シークエンスと系統発生解析により、フレボウイルス属に属する新規ウイルスを同定した。コッホの原則は完全には満たされなかったが、この米国における新たなフレボウイルス(ハートランドウイルス)はこれらの臨床的な症候の原因であると考えられる。

報告企業の意見	今後の対応
ミズーリ州でダニに刺され重症熱性疾患を発症した男性二人から新規フレボウイルスが検出されたとの報告である。	日本赤十字社では、輸血感染症対策として受付時に海外滞在歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、発熱などの体調不良者を献血不適としている。今後も引き続き情報の収集に努める。

使用上の注意記載状況・その他参考事項等

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



BRIEF REPORT

A New Phlebovirus Associated with Severe Febrile Illness in Missouri

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SUMMARY

Two men from northwestern Missouri independently presented to a medical facility with fever, fatigue, diarrhea, thrombocytopenia, and leukopenia, and both had been bitten by ticks 5 to 7 days before the onset of illness. *Ehrlichia chaffeensis* was suspected as the causal agent but was not found on serologic analysis, polymerase-chain-reaction (PCR) assay, or cell culture. Electron microscopy revealed viruses consistent with members of the Bunyaviridae family. Next-generation sequencing and phylogenetic analysis identified the viruses as novel members of the phlebovirus genus. Although Koch's postulates have not been completely fulfilled, we believe that this phlebovirus, which is novel in the Americas, is the cause of this clinical syndrome.

From the Viral Special Pathogens Branch (L.K.M., A.M., C.G.A., P.E.R., S.T.N.) and the Infectious Disease Pathology Branch (C.S.G., M.G.M., B.C.B., S.R.Z.), Division of High-Consequence Pathogens and Pathology, and the Rickettsial Zoonoses Branch, Division of Vector-Borne Diseases (A.J.K., W.L.N.), Centers for Disease Control and Prevention, Atlanta; and Heartland Regional Medical Center, St. Joseph, MO (S.M.F). Address reprint requests to Dr. Nichol at the Viral Special Pathogens Branch MS G14 or to Dr. Nicholson at the Rickettsial Zoonoses Branch MS G13, Centers for Disease Control and Prevention, 1600 Clifton Rd. NE, MS G-14, Atlanta, GA 30333, or at snichol@cdc.gov or wnicholson@cdc.gov.

Drs. McMullan and Folk contributed equally to this article.

N Engl J Med 2012;367:834-41.
DOI: 10.1056/NEJMoa1203378

THE PHLEBOVIRUS GENUS CONTAINS MORE THAN 70 ANTIGENICALLY DISTINCT viruses, which are divided into virus complexes according to whether they are borne by sand flies, mosquitoes, or ticks.[1] Sand-fly–borne viruses are found in the Americas, Asia, Africa, and the Mediterranean region, and infection with these viruses commonly results in a self-limiting 3-day fever, with the exception of Toscana virus, which can cause aseptic meningitis.[2] The prototype mosquito-borne phlebovirus is Rift Valley fever virus, which causes large-scale epizootics; human infection is often a self-limiting febrile illness that can progress to hepatitis, encephalitis, or hemorrhagic fever.[3] The only tickborne phlebovirus known to cause human disease is severe fever with thrombocytopenia syndrome virus (SFTSV), which was recently identified in central and northeastern China.[4]

CASE REPORTS

PATIENT 1

Patient 1 was a healthy 57-year-old man who lived on a 70-acre farm in northwestern Missouri. In early June 2009, he noticed a small nymphal tick embedded on his abdomen. The tick was subsequently removed with tweezers. There was no rash or localized itching. The following day, fever developed, which was followed by severe fatigue, headache, anorexia, nausea, and nonbloody diarrhea. Four days later, he was admitted to the hospital with a temperature of 37.9°C, which increased to 39.1°C the next day. Laboratory tests revealed a low white-cell count of 1900 cells per cubic millimeter, a low platelet count of 115,000 cells per cubic millimeter, and a low sodium level of 132 mmol per liter. Serum levels of liver aminotransferases were

slightly elevated, with an alanine aminotransferase level of 57 U per liter and an aspartate aminotransferase level of 44 U per liter. The serum level of C-reactive protein was elevated at 2.9 mg per deciliter. (Laboratory details are provided in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.)

The patient was hospitalized for 10 days. There was progression from moderate to severe thrombocytopenia, with a nadir of 37,000 cells per cubic millimeter on day 5 and 40,000 cells per cubic millimeter on days 6 and 7. Leukopenia continued throughout the hospitalization, with notable lymphopenia and mild neutropenia that progressed to moderate neutropenia on day 7 (Fig. 1A). Band forms were detected on days 2 and 8. An erythrocyte sedimentation rate was within the normal range at 9 mm per hour, and the erythrocyte count and hemoglobin were unremarkable and stable. The hematocrit was slightly low during hospitalization (Fig. 1C). The prothrombin time and partial-thromboplastin time were normal.

Serum hepatic aminotransferase levels increased and peaked, with an alanine aminotransferase level of 315 U per liter and an aspartate aminotransferase level of 431 U per liter on day 8 (Fig. 1B). Serum alkaline phosphatase levels rose within normal limits and peaked at 101 U per liter on day 9. Levels of creatinine and blood urea nitrogen remained normal. Urinalysis showed trace protein and 1+ ketones and was otherwise normal. Serum albumin levels were low, and serum sodium and calcium levels were mildly low.

On the second day of hospitalization, blood was sent to the Rickettsial Zoonoses Branch of the Centers for Disease Control and Prevention (CDC) and was subsequently shown to be negative for *E. chaffeensis*, *E. ewingii*, and rickettsiae of the spotted fever group on PCR assay. Serologic analysis later confirmed negative results of IgM and IgG assays for the spotted fever group and typhus. A rapid test for influenza A and B antigens was negative (Meridian Bioscience). Two blood cultures were sterile.

The patient was empirically placed on doxycycline (100 mg) intravenously twice daily for 14 days for suspected ehrlichiosis. Nonbloody diarrhea persisted through the fourth day of hospitalization. Stool specimens were negative for leukocytes, *Clostridium difficile* toxins, and salmonella, shigella, and campylobacter species. The results of two-dimensional echocardiography and chest radiography were normal.

The patient has reported fatigue and recurrent headaches in the 2 years since his hospitalization, but these symptoms cannot be clearly attributed to the viral infection. In addition, he initially had short-term memory difficulty, which has slowly improved, and anorexia, which resolved 4 to 6 weeks after discharge.

PATIENT 2

Patient 2 was a 67-year-old man with a 5-year history of type 2 diabetes who was otherwise healthy. He lived on an approximately 100-acre farm in northwestern Missouri. While on his property in early 2009, he received an average of 20 tick bites daily for approximately 2 weeks. He removed the embedded ticks with his fingers and tweezers. The last tick bite was noticed 1 week before hospitalization. Approximately 4 days before hospitalization, subjective fever, fatigue, and anorexia developed. Additional symptoms included myalgia, dry cough, and nonbloody diarrhea. No rash was noted before or during hospitalization.

On hospital admission in June 2009, his temperature was 38.1°C and reached 39.1°C the following day. Laboratory studies that were conducted on admission showed a low white-cell count of 2100 cells per cubic millimeter, a low platelet count of 78,000 cells per cubic millimeter, and a slightly elevated aspartate aminotransferase level of 54 U per liter (Fig. 1A, 1B, and 1C). The serum sodium level was slightly low at 130 mmol per liter, as was the calcium level at 7.8 mg per deciliter (1.95 mmol per liter). The results of urinalysis were normal.

The patient was hospitalized for 12 days. After day 2, thrombocytopenia progressed from moderate to severe, with a nadir of 34,000 cells per cubic millimeter on days 5 and 6. Platelet numbers increased starting on day 8 and reached a normal level by day 11 (Fig. 1C). Testing for antiplatelet antibodies was negative. Leukopenia continued until day 10, with mild neutropenia progressing to moderate neutropenia on days 6 to 8 (Fig. 1A). Band forms were present on days 2 to 7, and lymphocytes gradually increased to a normal range by day 8 (Fig. 1A). Erythrocyte counts and hemoglobin were within normal limits, and the hematocrit was slightly low throughout hospitalization. The prothrombin time, partial thromboplastin time, and fibrinogen levels were nor-

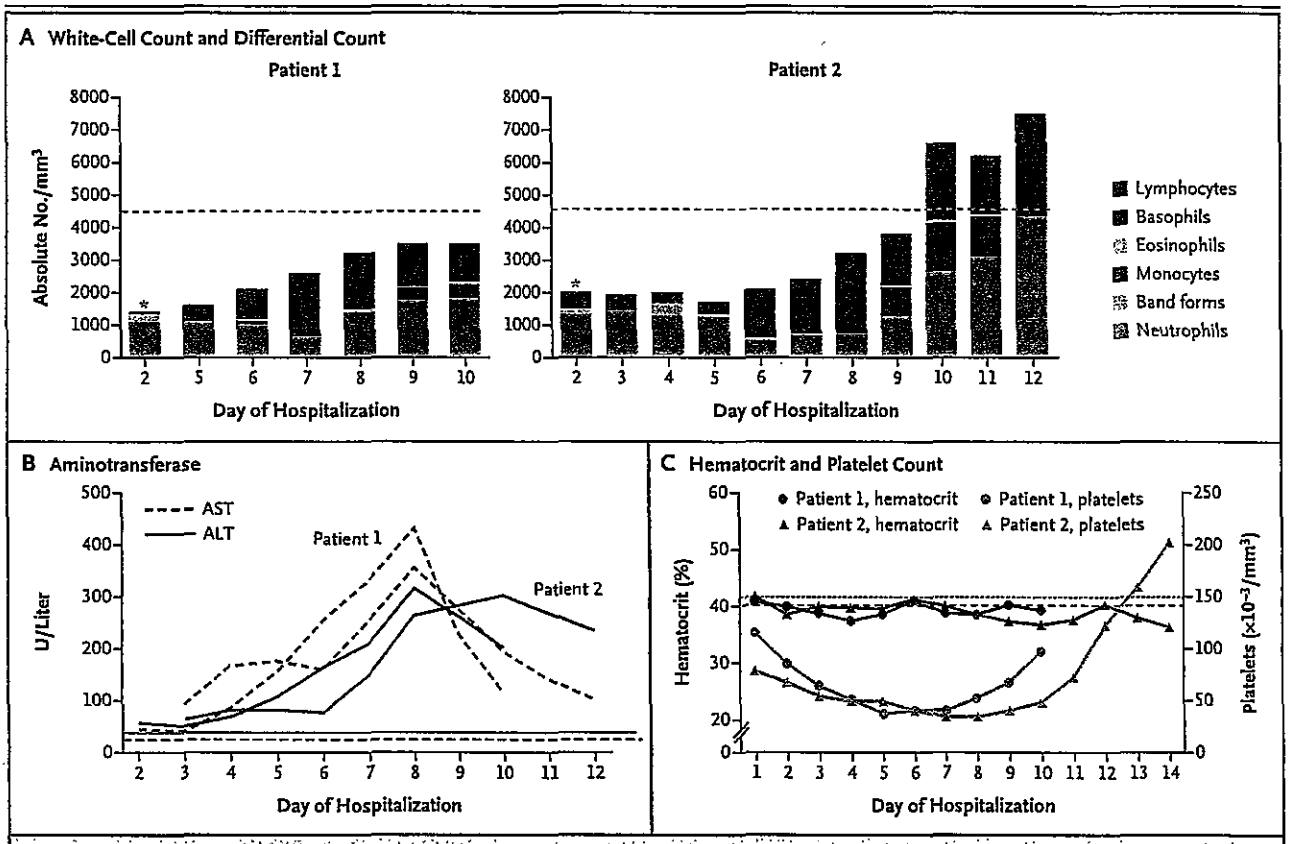

Figure 1. Laboratory Values for Patients 1 and 2.

Panel A shows the absolute values for the white-cell count and differential count for Patients 1 and 2 during hospitalization. The dashed lines indicate normal values. The asterisks indicate the day on which the novel virus was isolated from leukocytes obtained from the patients. Panel B shows levels of alanine aminotransferase (ALT; solid lines) and aspartate aminotransferase (AST; dashed lines) for the patients during hospitalization. The gray lines indicate normal values. Panel C shows hematocrit values (green) and platelet counts (gold) during the patients' hospitalization. The dashed lines indicate normal values.

mal, but the serum D-dimer level was elevated, at 4.08 mg per liter.

Blood was collected on day 2 of hospitalization and sent to the CDC. PCR results were found to be negative for *E. chaffeensis* and a range of ehrlichia and anaplasma species. Testing that was specific for borrelia antibody (Quest Diagnostics) was negative.

Alanine and aspartate aminotransferase levels were elevated and increased to 355 U per liter on day 8 and 302 U per liter on day 10, respectively (Fig. 1B). The alkaline phosphatase level was temporally high on day 10 but then resumed normal levels. Levels of creatinine and blood urea nitrogen remained normal. Levels of serum albumin and sodium remained low throughout hospitalization. Low serum calcium levels increased to normal by day 10. Results on chest radiography and abdominal ultrasonography were normal.

A bone marrow aspiration and biopsy were performed on day 2 of hospitalization. Trilineage hematopoiesis was detected, with less than 1% blasts and no ringed sideroblasts. There was notable defective development of erythrocytes (dyserythropoiesis) and megakaryocytes (dysmegakaryocytopoiesis). Flow cytometry confirmed 3 to 4% plasma cells with monoclonal lambda restriction, indicating response to infection. Cultures for fungi and mycobacteria were sterile.

The patient was initially treated empirically with intravenous piperacillin–tazobactam and was switched to ceftriaxone on hospital day 2 and to oral doxycycline (100 mg) twice daily on day 3 for suspected ehrlichiosis. He completed a 14-day course of doxycycline.

After hospital discharge, the patient noted fatigue, short-term memory difficulty, and anorexia. All the symptoms abated after 4 to 6 weeks and have not recurred in 2 years. Six months after discharge, the CDC confirmed the patient was negative for *E. chaffeensis* and *Anaplasma phagocytophilum* on IgG assay.

METHODS

CLINICAL SPECIMENS AND VIRUS CULTURE

EDTA-treated blood was collected and leukocytes separated with the use of Ficoll histopaque gradients and inoculated onto the canine monocyte cell line DH82.[5] Adherent and nonadherent cells were examined with the use of a modified rapid Wright–Giemsa stain (Diff-Quik). Culture supernatant was collected and transferred to Vero E6 cells and LLC-MK2 cells for virus propagation.

VIRUS GENOME SEQUENCING

Total RNA was extracted from infected cell culture media with the use of TriPure (Roche) and RNeasy (Qiagen) columns and nonspecifically amplified by means of random primers in a one-step reverse-transcriptase PCR reaction (SSIII RT–Platinum Taq HiFi Enzyme Mix, Invitrogen). Complementary DNA products were sequenced by means of next-generation sequencing (Roche 454) and analyzed with the use of bioinformatics tools.[6] (Details are provided in the Supplementary Appendix.)

RESULTS

ISOLATION OF A VIRUS FROM PATIENT LEUKOCYTES

Leukocytes were collected from both patients on day 2 of hospitalization and inoculated onto cultures of DH82 cells. These cultures showed cytopathic effects similar to early cultures of *E. chaffeensis*. However, cellular vacuoles did not contain bacterial morulae. Transfer of culture supernatants onto fresh DH82 cells resulted in similar cytopathic effects within 9 to 11 days. Cytopathic effects were less evident but also noted in Vero E6 cells 9 days after inoculation.

Studies were initiated to identify the suspected virus. Thin-section electron microscopy revealed enveloped particles averaging 86 nm in diameter, typical of a virus in the Bunyaviridae family (Fig. 2).

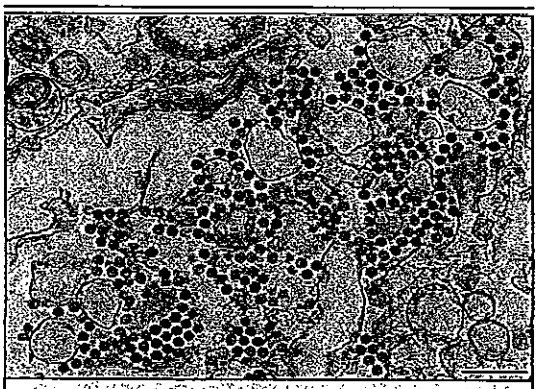

Figure 2. Thin-Section Electron Microscopy of Vero E6 Cells Revealing Virus Particles.

Extracellular enveloped, spherical virus particles with fairly homogeneous cores are visible in Vero E6 cells that were fixed in glutaraldehyde and processed for thin-section electron microscopy. Scale bar indicates 500 nm.

GENETIC ANALYSIS OF A NOVEL PHLEBOVIRUS

Total RNA was isolated from infected culture media and subjected to next-generation sequencing. The resulting full-length genome sequences were found to be similar to those of phleboviruses in the Bunyaviridae family, which are single-stranded, negative-sense RNA viruses comprised of three genome segments. We called this newly discovered virus the Heartland virus.

The phleboviruses share a similar genome organization.[7] The L segment is 6.4 kb in length and encodes a large RNA-dependent RNA polymerase. The M segment is 3.4 kb in length and encodes a polyprotein processed into the virus glycoproteins Gn and Gc, which are used for virion entry and assembly. The S segment is 1.7 kb in length and encodes the nucleoprotein that encapsidates the genomic RNA and a nonstructural (NSs) protein in an ambisense coding strategy. The genomes of virus isolates from both patients were sequenced in their entirety and found to be closely related, with 98%, 95%, and 99% identity for the S, M, and L virus segments, respectively. The high genetic identity indicates that both patients were infected with the same phlebovirus strain, but the differences between the isolates suggest that the two patients were infected independently.

PHYLOGENETIC ANALYSIS

Phylogenetic analysis of the aligned amino acid sequence of the polymerase, glycoprotein, nucleo-

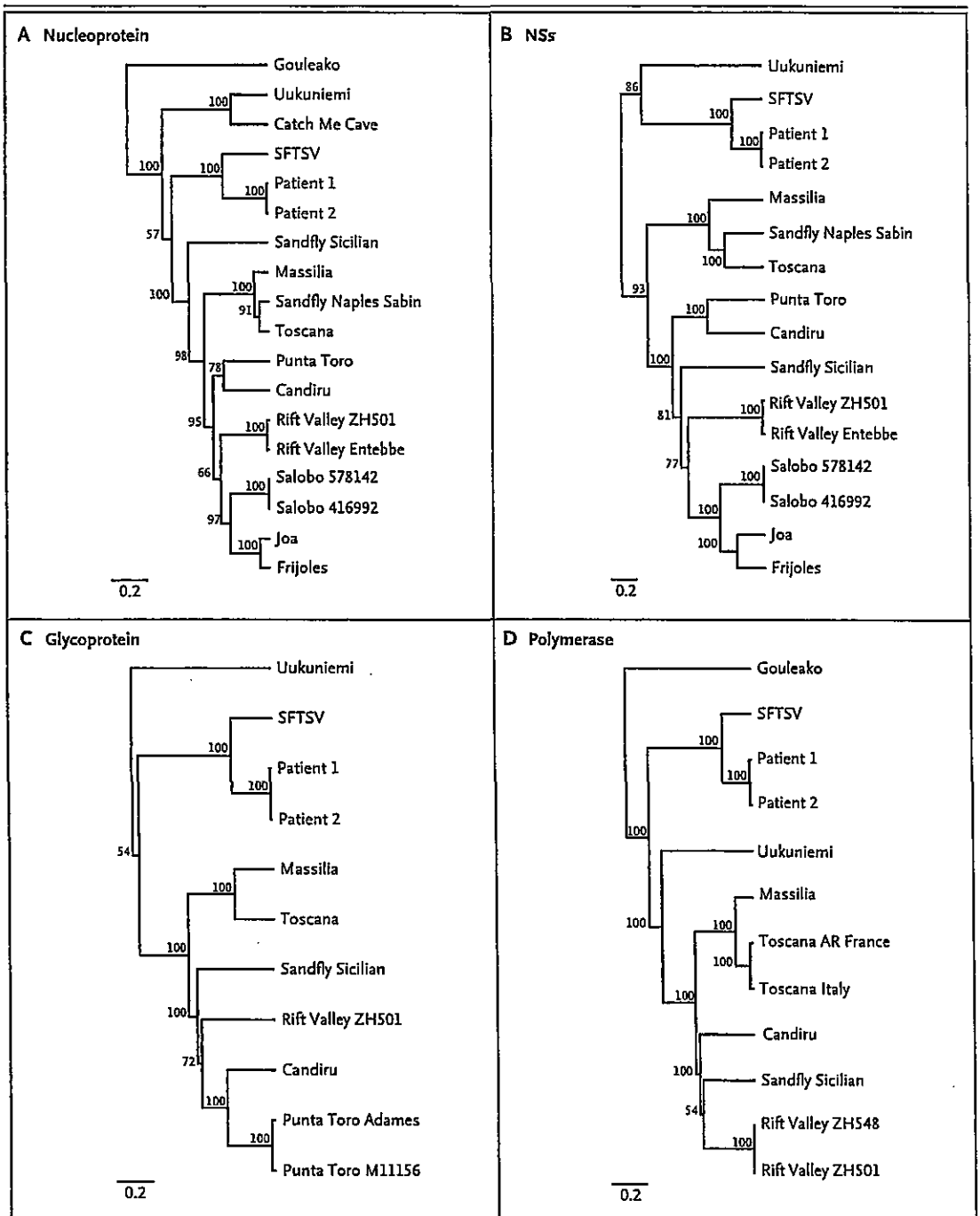

Figure 3. Phylogenetic Analyses of Amino Acid Sequencing of Representative Members of the Phlebovirus Genus.
The novel virus that was identified in Patients 1 and 2, a member of the phlebovirus genus, clusters with the tick-borne viruses and is most closely related to the severe fever with thrombocytopenia syndrome virus (SFTSV), which was recently identified in China. Analyses included sequencing of nucleoprotein (Panel A), nonstructural protein NSs (Panel B), glycoprotein (Panel C), and polymerase (Panel D). Phlebovirus sequences of each virus protein were aligned with the use of multiple alignment with fast Fourier transform (MAFFT), and phylogenic relationships were inferred with the use of the unweighted pair group method with arithmetic mean (UPGMA) method with 2000 bootstrap replicates for statistical support (CLC Genomics, Geneious). The GenBank accession numbers for the three segments for virus isolates from the two patients are provided in the Supplementary Appendix.

protein, and NSs protein suggested that the novel virus is a distinct member of the phlebovirus genus, clustering with the tickborne viruses and most closely related to SFTSV[4,8] (Fig. 3). This relationship is distant; however, pairwise comparisons of the viral polymerase and nucleoprotein (the two most conserved virus proteins) showed differences of 27% and 38%, respectively. Greater differences are found among the phlebovirus complexes that are borne by ticks, sand flies, and mosquitoes, which differ by at least 35%.

The novel virus was also distinct from an uncharacterized bunyavirus called lone star virus, which was isolated in 1967 from a nymphal *Amblyomma americanum* tick found on a woodchuck in western Kentucky.[9] Comparison of the polymerase amino acid sequence showed that lone star virus shared only 34% identity with the novel virus.

VIRAL RNA AND ANTIGEN IN BONE MARROW SPECIMEN

RNA of the novel virus was detected in bone marrow aspirate obtained from Patient 2. Immunohistochemical staining revealed the virus nucleocapsid protein in large mononuclear cells that did not resemble mature granulocytes, erythroid cells, or megakaryocytes. Staining was primarily cytoplasmic and seen in association with fragmented nuclear debris, which was a prominent finding in the biopsy specimen. No immunostaining was seen in control bone marrow–biopsy specimens or in normal rabbit serum used as a negative control (Fig. 4, and the Methods section in the Supplementary Appendix).

LONG-TERM PRESENCE OF REACTIVE IgG ANTIBODY

Patient serum samples were tested for the presence of antibodies reactive to the novel virus. In October 2011, more than 2 years after the onset of infection, blood was collected from both patients and serum samples were tested on enzyme-linked immunosorbent assay (ELISA) to detect IgG reactive with virus antigen (inactivated virus-infected cell lysate). Both serum samples were strongly positive, with titers of more than 6400.

DISCUSSION

Although Koch's postulates have not been completely fulfilled, our findings are consistent with the identification of a new pathogenic virus in the United States. This novel virus (which we called the Heartland virus) is a distinct member of the phlebovirus genus and is most closely related to tickborne phleboviruses, notably the recently isolated SFTSV. Clinical evaluations of the illness in the two patients who are described here probably do not reflect the entire spectrum of symptoms associated with this virus, yet both patients had a similar clinical course. Symptoms in the two patients included fever, fatigue, anorexia, and diarrhea.

Common laboratory findings were leukopenia with moderate neutropenia, thrombocytopenia, and elevated hepatic aminotransferase levels. Both patients had viremia on day 2 of hospitalization, approximately 7 days after the onset of symptoms. The temporal trends in white-cell and platelet counts and in aminotransferase levels were also strikingly similar between the two patients. Both patients presented with neutropenia that continued to decline to levels below 700 cells per cubic millimeter on days 6 and 7 of hospitalization. Thrombocytopenia continued until day 7 for both patients. Initially, the aminotransferase levels were only slightly elevated but spiked on days 7 and 8. After this time, there were increased levels of circulating neutrophils, lymphocytes, monocytes, and platelets, and aminotransferase levels began to normalize. Clinical evidence did not suggest respiratory or kidney involvement in either patient.

Many of the clinical and laboratory facets of this illness are similar to those reported for the tickborne phlebovirus SFTSV.[2] However, we did not observe coagulation abnormalities despite a markedly low platelet count, whereas a minority of patients with SFTSV infection had an elongated partial thromboplastin time and thrombin time, an elevated fibrinogen level, and symptoms of gingival bleeding and hemorrhage, with fatalities from disseminated intravascular coagulation and cerebral hemorrhage.[10,11] There have been numerous reports of person-to-person transmission on exposure to SFTSV-infected blood, and SFTSV has been detected in blood, throat swabs, urine, and feces obtained from patients with the infection.[10-13] It remains to be determined whether the novel virus can be transmitted from person to person, since no family members or caregivers of either patient reported symptoms resembling those of the patients. It will be important to determine how patients acquire this viral infection in order to promote risk-reduction practices.

The clinical laboratory results, symptoms, and

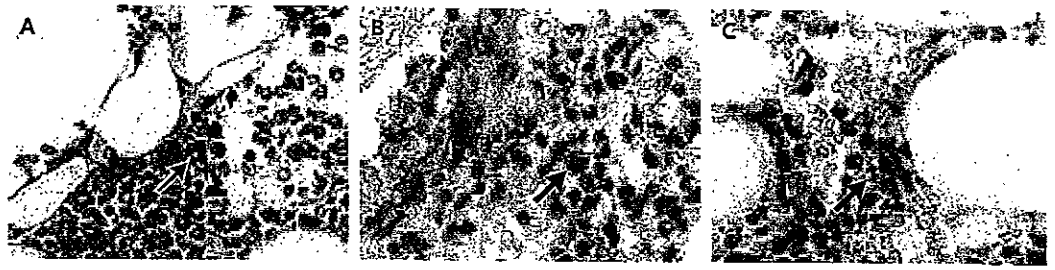

Figure 4. Histopathological and Immunohistochemical Staining of a Bone Marrow–Biopsy Sample from Patient 2.
Panel A shows fragmented nuclear debris (arrow) (hematoxylin and eosin). Panel B shows immunostaining of nucleocapsid protein from the novel phlebovirus within cytoplasm of large mononuclear cells (arrow). Panel C shows a higher-power image of the same sample indicating a granular immunostaining pattern (arrow). An indirect immunoalkaline phosphatase assay with naphthol fast-red substrate and light hematoxylin counterstaining was performed with the use of a 1:1000 dilution of hyperimmune rabbit serum reactive with the nucleoprotein of the novel phlebovirus (indicated in red).

occurrence of tick bite are similar to those of ehrlichiosis infections.[5] The novel virus should be considered as a possible etiologic agent in these instances, particularly when suspected ehrlichiosis does not improve within a few days of doxycycline treatment.

Although we did not isolate the novel virus from ticks, and tick specimens from the patients were not available, one potential vector is the lone star tick, *A. americanum*. Recent ecologic studies in central and southern Missouri found that 99.9% of captured ticks were *A. americanum*.[14] *A. americanum* is abundant in northwestern Missouri and found throughout the southeastern and south–central United States, extending up the Atlantic coast to Maine.[15] Both patients resided in areas with fragmented deciduous forest and old fields, suitable habitats for *A. americanum*. Studies will be required to determine the vector and potential hosts of this virus.

Although these two patients had severe disease, the incidence of infection with the novel virus and range of disease severity are currently unknown. Given the largely nonspecific symptoms observed, this virus could be a more common cause of human illness than is currently recognized. Epidemiologic and ecologic studies are needed to identify disease burden, risk factors for infection, and natural hosts of this new virus.

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the CDC.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Christopher Paddock and Jana Ritter for their consultation on histopathological and immunohistochemical analyses, Amy Denison for extraction and quality assessment of nucleic acids from bone marrow–biopsy samples, Mike Flint and Anita McElroy for discussions and editorial assistance in the preparation of the manuscript, and Ute Ströher and the Viral Special Pathogens Branch diagnostics group for providing ELISA data on viral IgG.

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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	新医薬品等の区分	総合機構処理欄
				2012. 9. 19	該当なし	

一般的名称	解凍人赤血球濃厚液	研究報告の公表状況		公表国
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社) 解凍赤血球液-LR「日赤」(日本赤十字社) 照射解凍赤血球液-LR「日赤」(日本赤十字社)		HealthDay News. Available from: http://consumer.healthday.com/Article.asp?AID=668809	米国

研究報告の概要

○ウエストナイル患者はまだ増加中;死亡者数は現在134人
米国のウエストナイルウイルス(WNV)感染患者数と死亡者数は増加し続け、9月19日時点で、患者数は合計3,142人、そのうち134人は死亡している。これは、米国における蚊媒介性疾患アウトブレイクの中でも最悪の事態である。米国CDCによると、アラスカ州とハワイ州を除く全ての州でヒト、動物または蚊にWNV感染が報告され、患者数の2/3は7州(カルフォルニア、ルイジアナ、ミシガン、ミシシッピ、オクラホマ、サウスダコタ、テキサス)から報告されている。全患者数のうち約40%はテキサス州から報告された。
全患者のうち、1,630人(52%)は髄膜炎や脳炎などの神経侵襲性疾患、1,512人(48%)は神経侵襲性疾患ではなかった。
米国でWNV感染患者が報告された2003年以降、9月の第3週までにCDCに報告された患者数としては最も多くなっている。

使用上の注意記載状況・その他参考事項等

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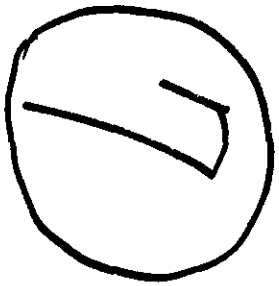
照射解凍赤血球液-LR「日赤」

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
米国におけるウエストナイルウイルス感染患者数及び死亡者数が増加し続け、2012年9月19日時点で合計3,142人となり、そのうち134人は死亡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受付時に海外滞在歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、ウエストナイルウイルス感染の国内発生に備え、平成17年10月25日付血液対策課発事務連絡に基づく緊急対応(献血制限、NAT検査)のほか、厚生労働科学研究「血液製剤の安全性確保と安定供給のための新興・再興感染症の研究」班と共同して対応について検討している。今後も引き続き情報の収集に努める。



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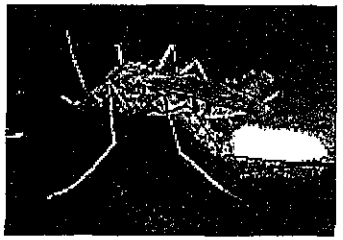
Texas continues to be epicenter of outbreak, with nearly 40 percent of all mosquito-borne infections

By Steven Reinberg
HealthDay Reporter



WEDNESDAY, Sept. 19 (HealthDay News) -- West Nile virus infections and deaths continue to climb, federal health officials reported Wednesday, with a new total of 3,142 cases and 134 deaths.

This makes it one of the worst outbreaks of the mosquito-borne disease ever to hit the United States.

While all states except Alaska and Hawaii have reported West Nile virus infections in humans, animals or mosquitoes, two-thirds of the human cases having been reported from seven states -- California, Louisiana, Michigan, Mississippi, Oklahoma, South Dakota and Texas, according to the U.S. Centers for Disease Control and Prevention.

Almost 40 percent of all cases have been reported in Texas.

Of the total number of cases, 1,630 (52 percent) have been classified as neuroinvasive disease (such as meningitis or encephalitis) and 1,512 (48 percent) have been classified as non-neuroinvasive disease, the CDC said.

The 3,142 cases reported so far in 2012 is the highest number of West Nile cases reported to the CDC through the third week of September since 2003.

The best way to avoid the virus is to wear insect repellant and support local programs to eradicate mosquitoes. There is currently no treatment for West Nile virus and no vaccine to pr[illegible]nt it, according to the CDC.

Typically, 80 percent of people infected with the virus develop no or few symptoms, while 20 percent develop mild symptoms such as headache, joint pain, fever, skin rash and swollen lymph glands, according to the CDC.

Although most people with mild cases of West Nile virus will recover on their own, the CDC recommends that anyone who develops symptoms see their doctor right away.

People older than 50 and those with certain medical conditions, such as cancer, diabetes, hypertension, kidney disease and organ transplants, are at greater risk for serious illness.

The best way to protect yourself from West Nile virus is to avoid getting bitten by mosquitoes, which can pick up the disease from infected birds.

The CDC recommends the following steps to protect yourself:

- Use insect repellents when outside.
- Wear long sleeves and pants from dawn to dusk.
- Don't leave standing water outside in open containers, such as flowerpots, buckets and kiddie pools.
- Install or repair windows and door screens.
- Use air conditioning when possible.

More information

For more on West Nile virus, visit the U.S. Centers for Disease Control and Prevention.

SOURCES: Sept. 19, 2012, statistics, U.S. Centers for Disease Control and Prevention

Last Updated: Sept. 19, 2012

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
				2012. 9. 19	該当なし	

一般的名称	解凍人赤血球濃厚液	研究報告の公表状況	Zbinden A, Mueller NJ, Tarr PE, Eich G, Schulthess B, Bahlmann AS, Keller PM, Bloemberg GV. J Clin Microbiol. 2012 Sep;50(9):2969-73. doi: 10.1128/JCM.00849-12. Epub 2012 Jul 3.	公表国
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研究報告の概要

○侵襲性感染症を引き起こす、*Streptococcus mitis*グループの新たな菌種*Streptococcus tigurinus*

*Strestococcus mitis*グループに属する新種である*Streptococcus tigurinus*(*S.tigurinus*)が感染性心内膜炎患者から分離された。表現型及び分子学的な特徴より、*S.tigurinus*は*S.mitis*、*S.pneumoniae*、*S.oralis*、*S.infantis*に非常に近い菌種である。

この研究で臨床感染症における*S.tigurinus*の関連及び*S.mitis*グループの菌種が口腔内に存在することから、口腔内における*S.tigurinus*の存在について評価した。

2003～2012年の期間に得られた臨床サンプルの16S rRNA遺伝子配列データベースを後方視的に分析したところ、14人の患者の通常無菌部位(血液、脳脊髄液または心臓弁)からS.tigurinusであると思われる17件の16S rRNA配列を検出した。これらの患者は重症侵襲性感染症に罹患していた(感染性心内膜炎6人、脊椎椎間板炎3人、菌血症2人、髄膜炎1人、人工関節の感染1人、膿胸1人)。

また、ボランティア31人の唾液サンプルをスクリーニングし、継代培養が可能であったα-溶血性細菌のコロニー608株(17人から採取)のうち26株がMALDI-TOF質量分析スクリーニングによって*S.tigurinus*であると示唆された。しかし16S rRNA遺伝子分析では、*S.tigurinus*に分類できる株は1つもないことが示された。*S.tigurinus*が口腔内細菌叢の一部として存在することは稀であるようだ。

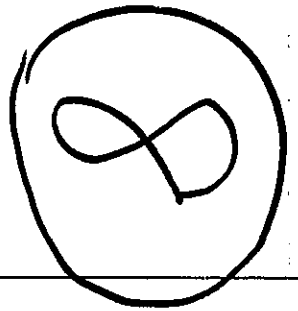
しかしながら、今回のデータは*S.tigurinus*が重要なヒトの病原体であることを実証する。*S.tigurinus*が分離された全ての患者が重症侵襲性感染症に罹患していたことは、侵襲性感染症(特に感染性心内膜炎)における*S.tigurinus*の潜在的な病原性を明らかにするために更なる研究が必要であることを示している。

報告企業の意見	今後の対応
侵襲性感染症の患者から、*Streptococcus mitis*グループの新種*Strestococcus tigurinus*が検出されたとの報告である。	日本赤十字社では全献血者に問診を実施しているほか、輸血による細菌感染予防対策として全輸血用血液製剤を対象に保存前白血球除去を行っている。また、輸血情報リーフレット等により、細菌感染やウイルス感染について医療機関へ情報提供し注意喚起しているほか、細菌感染が疑われる場合の対応を周知している。細菌やウイルスの検出や不活化する方策について検討している。

使用上の注意記載状況・その他参考事項等

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



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Streptococcus tigurinus, a Novel Member of the *Streptococcus mitis* Group, Causes Invasive Infections

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We recently described the novel species *Streptococcus tigurinus* sp. nov. belonging to the *Streptococcus mitis* group. The type strain AZ_3a^T of *S. tigurinus* was originally isolated from a patient with infective endocarditis. According to its phenotypic and molecular characteristics, *S. tigurinus* is most closely related to *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus oralis*, and *Streptococcus infantis*. Accurate identification of *S. tigurinus* is facilitated by 16S rRNA gene analysis. We retrospectively analyzed our 16S rRNA gene molecular database, which contains sequences of all clinical samples obtained in our institute since 2003. We detected 17 16S rRNA gene sequences which were assigned to *S. tigurinus*, including sequences from the 3 *S. tigurinus* strains described previously. *S. tigurinus* originated from normally sterile body sites, such as blood, cerebrospinal fluid, or heart valves, of 14 patients and was initially detected by culture or broad-range 16S rRNA gene PCR, followed by sequencing. The 14 patients had serious invasive infections, i.e., infective endocarditis ($n = 6$), spondylodiscitis ($n = 3$), bacteremia ($n = 2$), meningitis ($n = 1$), prosthetic joint infection ($n = 1$), and thoracic empyema ($n = 1$). To evaluate the presence of *Streptococcus tigurinus* in the endogenous oral microbial flora, we screened saliva specimens of 31 volunteers. After selective growth, alpha-hemolytic growing colonies were analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and subsequent molecular methods. *S. tigurinus* was not identified among 608 strains analyzed. These data indicate that *S. tigurinus* is not widely distributed in the oral cavity. In conclusion, *S. tigurinus* is a novel agent of invasive infections, particularly infective endocarditis.

We recently described a novel species within the *Streptococcus mitis* group, *Streptococcus tigurinus* sp. nov. (21). Based on phenotypic and molecular analyses, *S. tigurinus* is most closely related to *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus oralis*, and *Streptococcus infantis*. This novel species was not recognized in the past due to the limitations of conventional phenotypic test methods and of commercial systems (API 20 Strep and Vitek 2; bioMérieux, Marcy l'Etoile, France) as regards accurate identification of species within the *S. mitis* group (1, 3, 8, 11, 17). In addition, we have shown that *S. mitis* strain ATCC 15914 was initially misassigned when it was identified in 1977 (9); molecular analyses revealed the identification of strain ATCC 15914 as *S. tigurinus* (21). *S. tigurinus* colonies on sheep blood agar are alpha-hemolytic, smooth, and white to grayish with a diameter of 0.5 to 1 mm after incubation at 37°C with CO_2 for 24 h (21). Analyses by Vitek 2 resulted in identification as *S. mitis*/*S. oralis*, and analyses by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) revealed identification as *S. pneumoniae* with a score of ≥2.2 (21). However, the limited discriminative power of MALDI-TOF MS within the *S. mitis* group has been recognized previously by other authors (10, 18, 20). Hence, an identification result of *S. pneumoniae*, even with a score as high as ≥2.2, has to be interpreted with caution. Thus, analyses by commercial test systems, such as Vitek 2, or by MALDI-TOF MS are helpful for initial assignment to the *S. mitis* group, but genetic analyses are required for definitive assignment as *S. tigurinus*. We demonstrated a significant sequence demarcation within the 5′ part of the 16S rRNA gene (±500 bp) to the most closely related species, i.e., *S. mitis*, *S. pneumoniae*, *S. pseudopneumoniae*, and *S. oralis*, which allowed definite identification of *S. tigurinus* (21). This is remarkable because the discriminative power of the 5′ part of the 16S rRNA gene is not sufficient for accurate identification among these closely related species (1, 12).

With the description of *S. tigurinus*, we identified a novel pathogen associated with serious invasive infections. *S. tigurinus* was first documented as the causative agent in multiple blood cultures and aortic valve specimens of a patient with infective endocarditis (21). Other members of the *S. mitis* group, such as *S. mitis* and *S. oralis*, which are known agents of infective endocarditis (7, 17) and sepsis in neutropenic cancer patients (2, 15), are commensals of the oral flora. Therefore, the oral cavity is suspected to be the ecological niche of *S. tigurinus*.

The aim of our study was to determine the involvement of *S. tigurinus* in clinical infections and to assess the presence of *S. tigurinus* in the oral cavity. By retrospective analysis of our institute's 16S rRNA gene sequence database obtained from clinical samples covering the years 2003 to 2012, we identified *S. tigurinus* as an emerging pathogen causing invasive infections.

Received 29 March 2012 Returned for modification 3 May 2012
Accepted 25 June 2012
Published ahead of print 2 July 2012
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doi:10.1128/JCM.00849-12

MATERIALS AND METHODS

This study was approved by the ethics committee of the canton of Zurich, Switzerland. The study included a retrospective analysis of laboratory and clinical data and a prospective analysis of saliva specimens of volunteers.

Bacterial strains. A retrospective analysis covering the period 2003 to 2012 of the 16S rRNA gene sequence database (SmartGene, Zug, Switzerland) of the Institute of Medical Microbiology, University of Zurich, Switzerland, was performed. Bacterial strains were grown on Columbia agar plates containing 5% defibrinated sheep blood (bioMérieux) at 37°C under aerobic conditions.

Patients. Fourteen patients with *S. tigurinus* infections were hospitalized in Switzerland, and one patient in Germany. Clinical data were retrieved from the patients' medical records and reviewed by infectious disease specialists. Eleven patients were male; the mean age was 47.4 years (range, 21 to 74). Infective endocarditis was defined according to established criteria (14).

Analysis of 16S rRNA gene. DNA was extracted from the cultures as follows. A loopful of bacteria was suspended in 500 μl 0.9% NaCl and incubated by shaking at 80°C for 10 min. After centrifugation, the pellet was resuspended in 200 μl of InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) and incubated at 56°C for 2 h and then at 95°C for 10 min. The mixture was centrifuged, and the supernatant was used as the template for PCR. An 800-bp fragment of the 16S rRNA gene was amplified with primers BAK11w (5′-AGTTTGATCMTGGCTCAG-3′; positions 10 to 27, *Escherichia coli* numbering) and BAK2 [5′-GGACTACHAGGGTATCTAAT-3′; positions 806 to 787, *Escherichia coli* numbering]. The cycling parameters included an initial denaturation for 5 min at 95°C, 40 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C, and a final extension for 10 min at 72°C. Five microliters of the DNA extract was used for amplification in a total volume of 50 μl containing 1.25 U of AmpliTaq DNA polymerase LD (Applied Biosystems, Rotkreuz, Switzerland) and the appropriate buffer. Amplicons were purified with a QIAquick PCR purification kit (Qiagen AG, Hombrechtikon, Switzerland) and sequenced with the forward primer BAK11w using an automatic DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems). Broad-range 16S rRNA gene PCR was performed directly from clinical specimens as described earlier (4). 16S rRNA gene BLAST analysis was performed using SmartGene software (SmartGene, Zug, Switzerland). 16S rRNA gene sequences were stored in a Web-accessible database environment provided by SmartGene.

Antibiotic susceptibility testing. Antibiotic susceptibility testing was performed using susceptibility test disks (Becton, Dickinson, Germany, and i2a, Montpellier, France), and interpretation was done according to CLSI 2012 guidelines (6). For penicillin and high-level gentamicin resistance, MICs were determined using Etest strips (AB bioMérieux, Marcy l'Etoile, France). Susceptibility testing was performed on Mueller-Hinton agar supplemented with 5% sheep's blood, using overnight cultures at 0.5 McFarland standard followed by incubation at 35 ± 2°C with 5% CO_2 for 20 to 24 h.

Oral cavity specimens. Saliva samples were diluted with 0.85% NaCl and then plated on colistin-nalidixic acid agar. After incubation at 37°C with CO_2 for 24 h, alpha-hemolytic growing colonies were further analyzed. From each specimen, 20 morphologically different alpha-hemolytic colonies were picked and analyzed by MALDI-TOF MS after subcultivation. The analysis was performed by using a Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) using the MALDI Biotyper software package (version 3.0) with reference database version 3.1.2.0 (Bruker Daltonik GmbH). Sample preparation was done using the ethanol-formic acid extraction protocol according to the manufacturer's instructions. For the identification of *S. tigurinus*, reference spectra of the strains *S. tigurinus* AZ_3a^T and *S. tigurinus* AZ_8 were added to the MALDI-TOF reference database. All putative strains displaying both a score of >2.2 to *S. tigurinus* and a score of <2.0 to *S. pneumoniae* in the reference database were suggestive of *S. tigurinus*. For confirmation, 16S rRNA gene analysis was performed as described above.

Nucleotide sequence accession numbers and strain deposition. Partial 16S rRNA gene sequences of cultured and uncultured *S. tigurinus* strains have been deposited in GenBank under the following accession numbers: strain AZ_1 (GenBank accession number JQ696859), AZ_2 (JQ696860), AZ_3b (JQ696868), AZ_4a (JQ696861), AZ_4b (JQ696869), AZ_5 (JQ696870), AZ_6 (JQ696862), AZ_7a (JQ696863), AZ_7b (JQ696871), AZ_8 (JQ696864), AZ_9 (JQ696872), AZ_10 (JQ696865), AZ_11 (JQ696866), AZ_12 (JQ696867), AZ_13a (JQ696873), AZ_13b (JQ696874), AZ_14 (JQ778987), and AZ_15 (JQ820471). The following *S. tigurinus* strains have been deposited in the Culture Collection of Switzerland (CCOS, Wädenswil, Switzerland): AZ_1 (culture number CCOS 683), AZ_2 (CCOS 675), AZ_4a (CCOS 676), AZ_6 (CCOS 681), AZ_7a (CCOS 677), AZ_8 (CCOS 678), AZ_10 (CCOS 679), AZ_11 (CCOS 682), AZ_12 (CCOS 680), and AZ_14 (CCOS 689).

RESULTS

Retrospective analysis of 16S rRNA gene sequence database. By retrospective analysis of our institution's 16S rRNA gene sequence database, 17 sequences were identified which showed the highest 16S rRNA gene sequence similarities (99.4% to 100% identity) to *S. tigurinus* AZ_3a^T (JN004270). For all 17 sequences, BLAST search in GenBank revealed a sequence demarcation of ≥1.1% to the next validated species reference sequence. In general, a 16S rRNA gene sequence identity of ≥99.0% to a reference sequence of a classified species with a demarcation of >0.5% to the second classified species is considered to allow for assignment to species level (3). All 17 sequences, originally identified as *S. mitis* group, were assigned to *S. tigurinus*.

The 17 sequences were obtained from 14 patients and consisted of 12 *S. tigurinus* strains initially detected by culture methods, i.e., AZ_1, AZ_2, AZ_4a, AZ_6, AZ_7a, AZ_8, AZ_10, AZ_11, AZ_12, AZ_13a, AZ_13b, AZ_14, and 5 uncultured *S. tigurinus* bacteria detected by broad-range 16S rRNA gene PCR, i.e., AZ_4b, AZ_5, AZ_7b, AZ_9, and AZ_15. The *S. tigurinus* strains AZ_4a, AZ_7a, and AZ_10 have been described previously (21). The strains AZ_13a and AZ_13b were originally isolated at the Institute of Medical Microbiology, Friedrich-Schiller University of Jena, Germany. *S. tigurinus* bacteria identified from independent specimens of the same patient showed identical 16S rRNA gene sequences and are indicated with "a" and "b." *S. tigurinus* strains AZ_1, AZ_2, AZ_4a, AZ_6, AZ_7a, AZ_8, AZ_10, AZ_12, AZ_13a, and AZ_14 were originally cultured from blood, strain AZ_11 from a vertebral-body biopsy specimen, and strain AZ_13b from an aortic valve specimen, respectively. In 2 patients with *S. tigurinus* isolated from blood, a sibling *S. tigurinus* bacterium was also recovered from a heart valve (AZ_4b) and cerebrospinal fluid specimen (AZ_7b) by sequence analysis after broad-range 16S rRNA gene PCR. In three patients, *S. tigurinus* was detected only by broad-range 16S rRNA gene PCR, i.e., AZ_5 from a periarticular prosthetic hip biopsy specimen and AZ_9 and AZ_15 from mitral valves.

By analyzing the electropherogram of the 16S rRNA gene sequence of *S. tigurinus* strain AZ_1, double peaks were observed by sequencing with the forward primer BAK11w, indicating the presence of different 16S rRNA gene copies. In sequencing with the reverse primer BAK2, an unambiguous electropherogram signaling was obtained, consistent with a single copy. Detailed analysis of the electropherograms showed a frameshift mutation of two base pairs within the first 100 nucleotides of the 5′ part of the 16S rRNA gene, which resulted in a double-peak electropherogram

when sequencing with the forward primer. Other sequences did not show a double-peak electropherogram.

Patient clinical features. In total, we identified 15 patients with isolation of *S. tigurinus*, including the 4 patients of the original *S. tigurinus* species description (21). The patient characteristics are summarized in Table 1. All patients had serious infections. Seven patients were diagnosed with definite infective endocarditis according to the modified Duke criteria (14). *S. tigurinus* was isolated from multiple blood cultures in five patients; in three of these patients, the pathogen was also isolated from surgically resected heart valve specimens. In two patients with infective endocarditis, *S. tigurinus* was detected only in heart valve specimens, presumably due to antibiotic treatment before blood culture sampling. In one patient with bacterial meningitis, *S. tigurinus* was found in multiple blood cultures and in cerebrospinal fluid. *S. tigurinus* was associated with spondylodiscitis ($n = 3$), prosthetic joint infection ($n = 1$), and thoracic empyema ($n = 1$). Two patients had bacteremia alone. In 11 patients, *S. tigurinus* was the only organism detected in the specimens. Four patients had mixed infections, i.e., two patients with *S. tigurinus* and *Streptococcus salivarius* group bacteria, one patient with *S. tigurinus*, *S. salivarius* group bacteria, and a streptococcal species which was most closely related to *Streptococcus parasanguinis*, and one patient with *S. tigurinus* and *Staphylococcus aureus*. Most of the mixed infections occurred in patients with intravenous drug use. Both immunocompromised and immunocompetent patients had *S. tigurinus* infections. All patients recovered after appropriate antimicrobial therapy, with ($n = 7$) or without surgery ($n = 8$).

Antimicrobial susceptibility profile. The antibiotic susceptibility profile was determined for the cultured *S. tigurinus* strains ($n = 13$). All strains were susceptible to penicillin, and none of the strains displayed high-level gentamicin resistance (Table 2). For tetracycline, the strains AZ_6, AZ_7a, and AZ_14 displayed reduced susceptibility or resistance. No inducible clindamycin resistance was detected.

Frequency of *S. tigurinus* in causing endocarditis. Microbiological reports from the Institute of Medical Microbiology from 2003 to 2012 that were consistent with infective endocarditis were investigated. In total, we identified 48 cases of infective endocarditis caused by viridans group streptococci, 7 (14.5%) of which were caused by *S. tigurinus*. Thirteen (27%) endocarditis cases were caused by *S. mitis/S. oralis*, 11 (23%) by *Streptococcus sanguinis*, 6 (12.5%) by *Streptococcus gordonii*, and the remaining 11 (23%) endocarditis cases were caused by other viridans group streptococci.

Screening for *S. tigurinus* in the oral cavity. Paraffin-stimulated saliva specimens of 31 volunteers (mean age of 27.8 years, range of 19 to 49 years; 16 male) were investigated for the presence of *S. tigurinus*. From the initial 620 alpha-hemolytic bacterial colonies isolated from the saliva of 31 persons, 12 strains did not grow after subcultivation, and therefore, 608 strains were analyzed by MALDI-TOF MS. Using MALDI-TOF screening criteria, 26 of the 608 strains, obtained from 17 persons, were suggestive for *S. tigurinus*. However, subsequent 16S rRNA gene analyses showed that none of the strains could be assigned to *S. tigurinus*; a single strain was closely related to *S. tigurinus* AZ_3a^T (JN004270), with a sequence similarity of 98.9%. Because the MALDI-TOF screening criteria, i.e., a score of >2.2 to *S. tigurinus* and a score of <2.0 to *S. pneumoniae*, might have been too strict for the detection of *S. tigurinus* strains, we tested an additional 21 strains which displayed a MALDI-TOF score of >2.2 to *S. tigurinus* and a score of ≥2.0 to *S. pneumoniae* by molecular methods. Even with these relaxed criteria, however, no *S. tigurinus* strains were detected.

For the most recent endocarditis patients with isolation of *S. tigurinus* (patients 14 and 15) (Table 1), we had the opportunity to test saliva specimens for the presence of *S. tigurinus*. For patient 14, the saliva specimen was obtained at day 13 of antibiotic therapy. None of the 20 strains investigated was suggestive for *S. tigurinus*. For patient 15, the analysis of the saliva specimen obtained at day 15 of antibiotic therapy revealed 2 strains suggestive for *S. tigurinus*, but they were not confirmed by molecular methods.

DISCUSSION

Using our institute's molecular database containing all 16S rRNA gene sequences obtained from clinical samples over a 10-year period, we showed the involvement of the newly described *S. tigurinus* species in serious clinical infections. Including the first recognized cases with *S. tigurinus* (21), we identified, in total, 15 patients from whom *S. tigurinus* was either isolated by initial culture methods or identified by broad-range 16S rRNA gene PCR. Most patients had infective endocarditis, but *S. tigurinus* was also identified as the causative agent in patients with spondylodiscitis, meningitis, prosthetic joint infection, thoracic empyema, and bacteremia. *S. tigurinus* infection was documented in both immunocompromised and immunocompetent patients of various ages and with a wide range of underlying conditions. Given the limited number of patients, however, a specific risk factor profile for the development of invasive infections with *S. tigurinus* could not be established. Data regarding the dental and mucosal health of the patients were limited by the retrospective study design.

Most patients with *S. tigurinus* infection had endocarditis. The frequency of *S. tigurinus* in causing infective endocarditis compared to the frequencies of other viridans group streptococci seems to be remarkably high, since 14.5% of all putative endocarditis cases associated with viridans group streptococci as observed at our institute during 2003 to 2012 were caused by *S. tigurinus*. The number of patients with infective endocarditis might even be higher due to missing information. The causative agents in 11 of the 13 endocarditis cases caused by *S. mitis/S. oralis* were identified only by conventional phenotypic methods, such as Vitek and API 20 Strep (bioMérieux). Therefore, it is possible that a substantial proportion of these bacteria actually represent *S. tigurinus* if molecular analyses had been performed.

The oral microbial flora was assumed to represent a main reservoir of *S. tigurinus*, similar to the case for other viridans group streptococci. However, screening a population of 31 volunteers for the presence of *S. tigurinus* in the saliva did not reveal any *S. tigurinus* strains. In two patients with infective endocarditis caused by *S. tigurinus*, we were able to evaluate saliva specimens during the first 15 days of antibiotic therapy. *S. tigurinus* was not detected in these specimens. *S. tigurinus* seems rarely to be present as part of the oral flora. Given the limited discriminative power of MALDI-TOF MS within the *S. mitis* group (10, 18, 20) and the limited capacity to analyze all strains by molecular methods, however, we might have underestimated the presence of *S. tigurinus* in the oral microbial flora. A BLAST search in GenBank revealed that *S. tigurinus* is likely to be present in the human oral cavity, since a number of 16S rRNA gene sequences deriving from the human mouth showed high sequence similarities to the *S. tigurinus* type strain sequence (JN004270), allowing accurate assignment (16).

TABLE 1 Characteristics of patients with *Streptococcus tigurinus* infections[a]

Patient ($n = 15$)	Age (yr), sex	*S. tigurinus* (strain/ uncultured)	Site of isolation (no. of positive blood cultures)	Immunosuppression	Comorbidity(ies)	Oral health status	Antibiotic treatment	Diagnosis	Outcome
1	47, M	AZ_1[b]	Blood (4)[c]	Systemic lupus erythematosus, long-term corticosteroid therapy	CAD, renal insufficiency, pure red cell aplasia	ND	AMC and RIF	Bacteremia	Recovered
2	58, M	AZ_2[b]	Blood (6)[c]	No	Prosthetic aortic valve	ND	VAN, CIP, and RIF, then PEN and GEN, and then CRO	Spondylodiscitis, bacteremia	Recovered
3	74, F	AZ_3a,[b] AZ_3b[d]	Blood (6)[c] and aortic valve	Heavy alcohol use	Aortic stenosis	Dental procedure 2 mo earlier	AMC and then PEN and GEN	Endocarditis	Recovered
4	37, F	AZ_4a,[b] AZ_4b[d]	Blood (6)[c] and mitral valve	No	No	No abnormalities detected	PEN and GEN and then CRO	Endocarditis	Recovered
5	65, M	AZ_5[d]	Periarticular hip biopsy[e]	Heavy alcohol use	Possible cirrhosis of the liver, atrial fibrillation	Extraction of several carious teeth during antibiotic therapy	CRO then CTX	Infection of prosthetic hip joint	Recovered
6	35, M	AZ_6[b]	Blood (4)[e]	No	Intravenous drug use	ND	AMC, then ERTA, and then AMC	Thoracic empyema, bacteremia	Recovered
7	30, M	AZ_7a,[b] AZ_7b[d]	Blood (4)[c] and cerebrospinal fluid	No	Cerebrospinal fluid leak after operation for hypophyseal tumor	ND	CRO, VAN, and MTZ and then CRO	*Meningitis*	Recovered
8	64, M	AZ_8[b]	Blood (8)[e]	No	No	No abnormalities detected	AMC and GEN and then PEN	Endocarditis	Recovered
9	65, M	AZ_9[d]	Mitral valve[e]	No	No	ND	CRO and then AMC and GEN	Endocarditis	Recovered
10	29, F	AZ_10[b]	Blood (2)[f]	No	Intravenous drug use	ND	CIP and then AMC and GEN	Spondylodiscitis, bacteremia	Recovered
11	32, M	AZ_11[b]	Vertebral body biopsy[g]	No	Intravenous drug use	ND	AMC	Spondylodiscitis	Recovered
12	50, M	AZ_12[b]	Blood (1)[g]	HIV infection	Myocardial infarction	ND	AMX	Bacteremia	Recovered
13	47, M	AZ_13a,[b] AZ_13b[b]	Blood (5),[c] aortic valve[e]	No	CAD	No abnormalities detected	CRO and AMC	Endocarditis, focal encephalitis	Recovered
14	57, M	AZ_14[b]	Blood (8)[e]	No	Hypertrophic obstructive cardiomyopathy	No abnormalities detected	PEN and GEN and then PEN	Endocarditis	Recovered
15	21, F	AZ_15[d]	Mitral valve[e]	No	No	Healthy teeth, mild gingivitis	PEN and GEN	Endocarditis	Recovered

[a] Abbreviations: CAD, coronary artery disease; ND, not determined; AMC, amoxicillin-clavulanic acid; AMX, amoxicillin; CTX, cefotaxime; CIP, ciprofloxacin; CRO, ceftriaxone; ERTA, ertapenem; GEN, gentamicin; MTZ, metronidazole; PEN, penicillin; RIF, rifampin; VAN, vancomycin.

[b] Cultured strain.

[c] *S. tigurinus* was the only organism detected in the specimens.

[d] Uncultured, detected by broad-range 16S rRNA gene PCR analysis.

[e] *Staphylococcus aureus* was detected in the same blood cultures.

[f] *Streptococcus salivarius* group bacteria and *Streptococcus* sp. bacteria most closely related to *Streptococcus parasanguinis* were detected in the same blood cultures.

[g] *Streptococcus salivarius* group bacteria were detected in the same specimen.

TABLE 2 Antimicrobial susceptibilities of the *Streptococcus tigurinus* strains[a]

Strain ($n = 13$)	Susceptibility (MIC [μg/ml]) of:		Antibiotic resistance disk test result (zone of inhibition [mm])				
	Penicillin	Gentamicin	LVX	VAN	ERY	CLI	TET
AZ_1	S (0.023)	S (24)	S (28)	S (24)	S (32)	S (32)	S (38)
AZ_2	S (0.023)	S (24)	S (26)	S (22)	S (29)	S (30)	S (34)
AZ_3a^T	S (0.047)	S (24)	S (25)	S (21)	S (27)	S (26)	S (30)
AZ_4a	S (0.047)	S (24)	S (27)	S (22)	S (30)	S (26)	S (25)
AZ_6	S (0.064)	S (32)	S (24)	S (23)	S (30)	S (30)	R (18)
AZ_7a	S (0.032)	S (16)	S (25)	S (24)	S (30)	S (26)	I (21)
AZ_8	S (0.032)	S (64)	S (27)	S (23)	S (30)	S (25)	S (34)
AZ_10	S (0.064)	S (64)	S (24)	S (23)	S (31)	S (26)	S (32)
AZ_11	S (0.012)	S (16)	S (27)	S (25)	S (34)	S (32)	S (34)
AZ_12	S (0.125)	S (32)	S (27)	S (25)	S (34)	S (28)	S (34)
AZ_13a	S (0.012)	S (4)	S (32)	S (27)	S (36)	S (34)	S (36)
AZ_13b	S (0.047)	S (12)	S (27)	S (26)	S (34)	S (26)	S (36)
AZ_14	S (0.047)	S (16)	S (27)	S (24)	S (30)	S (28)	I (20)

[a] The breakpoints of the 2012 CLSI guidelines were applied (6). Abbreviations: S, susceptible; R, resistant; I, intermediate; LVX, levofloxacin; VAN, vancomycin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline.

This is consistent with the mucosal presence of streptococcal species in general (17). In future, oral microbiome projects will provide a more detailed analysis of the composition of the oral microbial flora and the presence of *S. tigurinus*.

To date, we have detected *S. tigurinus* as the causative agent of invasive infections in patients from Switzerland and Germany, and it is likely that other bacterial agents previously reported as belonging to the *S. mitis* group actually represent *S. tigurinus*. This is most likely due to limitations of species identification within the *S. mitis* group by conventional means. In general, sequences of strains classified only by biochemical phenotypic test methods frequently result in deposition in GenBank with an inaccurate species assignment (5), e.g., the erroneous assignment of strain ATCC 15914 as *S. mitis* (13, 19, 21).

Our data clearly document that *S. tigurinus* is a significant human pathogen. The observation that all patients from whom *S. tigurinus* was isolated had severe invasive infections (Table 1) suggests the importance of conducting further studies to better characterize the potential pathogenic role of *S. tigurinus* in invasive infections, particularly infective endocarditis. A prerequisite is the accurate identification of viridans group streptococci by molecular methods. Further work also should focus on the natural habitat of and the potential for colonization with *S. tigurinus*.

ACKNOWLEDGMENTS

The study was supported by the University of Zurich.

We thank the laboratory technicians for their dedicated help. We thank R. Zbinden for constructive discussion and continuous support. We thank S. Christen, T. Bruderer, and M. Marchesi for substantial assistance.

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