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

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

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

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

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

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

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医薬品　研究報告　調査報告書

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販売名（企業名）	別紙のとおり				

研究報告の概要

問題点：脳出血で死亡した臓器提供者から腎臓と肝臓の提供を受けた後に死亡した3人の女性から発見されたウイルスは、遺伝子配列解析により、リンパ球性脈絡膜髄膜炎ウイルス様の新種のアレナウイルスと判明した。

2006年12月に3ヵ月間の前ユーゴスラビアの地方滞在からオーストラリアに帰国して10日後に脳出血で死亡した57才の臓器提供者から、腎臓と肝臓の提供を受けた63,64,44才の3人の女性が死亡した。女性たちは、移植直後の経過に変わったところはなかったが、その後脳症を伴う熱性疾患を発症し、提供を受けて4～6週後の2007年1月初めに死亡した。2人の患者から移植されたそれぞれの肝臓と腎臓のRNAの塩基配列を解析した結果、リンパ球性脈絡膜髄膜炎ウイルス（LCMV）様の新種のアレナウイルスと思われる遺伝子配列が検出された。また、PCR解析により、患者の腎臓、肝臓、血液及び髄液からウイルスの遺伝子断片が、免疫組織化学的解析により、移植された肝臓及び腎臓からウイルスの抗原が検出された。さらに、患者血清からは抗ウイルスIgM及びIgG抗体も検出された。

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

使用上の注意記載状況・その他参考事項等

記載なし

一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免役グロブリン、⑤乾燥ペプシン処理人免疫グロブリン、⑥乾燥スルホ化人免疫グロブリン、⑦乾燥スルホ化人免疫グロブリン*、⑧乾燥濃縮人活性化プロテインC、⑨乾燥濃縮人血液凝固第Ⅷ因子、⑩乾燥濃縮人血液凝固第Ⅸ因子、⑪乾燥抗破傷風人免疫グロブリン、⑫抗 HBs 人免疫グロブリン、⑬トロンビン、⑭フィブリノゲン加第ⅩⅢ因子、⑮乾燥濃縮人アンチトロンビンⅢ、⑯ヒスタミン加人免疫グロブリン製剤、⑰人血清アルブミン*、⑱人血清アルブミン*、⑲乾燥ペプシン処理人免役グロブリン*、⑳乾燥人血液凝固第Ⅸ因子複合体*、㉑乾燥濃縮人アンチトロンビンⅢ
販売名(企業名)	①献血アルブミン 20“化血研”、②献血アルブミン 25“化血研”、③人血清アルブミン“化血研”*、④“化血研”ガンマーグロブリン、⑤献血静注グロブリン“化血研”、⑥献血ベニロンーⅠ、⑦ベニロン*、⑧注射用アナクトC2,500 単位、⑨コンファクトF、⑩ノバクトM、⑪テタノセーラ、⑫ヘパトセーラ、⑬トロンビン“化血研”、⑭ボルヒール、⑮アンスロビンP、⑯ヒスタグロビン、⑰アルブミン 20%化血研*、⑱アルブミン 5%化血研*、⑲静注グロブリン*、⑳ノバクトF*、㉑アンスロビンP1500 注射用
報告企業の意見	LCMV は、アレナウイルス属に属するエンベロープに包まれた直径 30～300nm の不定形粒子であり、二種類のマイナス一本鎖 RNA を有する。げっ歯類を自然宿主とし、その糞尿や唾液、血液の曝露によってヒトに伝播する。LCMV 感染症は多くは無症候性あるいは軽度であるが、妊婦では無菌性髄膜炎や脳炎、致死性の感染症を起こす危険がある。臓器移植患者における LCMV のヒト-ヒト感染は、過去にも報告がある。 弊所の血漿分画製剤の製造工程には、冷エタノール分画工程、ウイルス除去膜ろ過工程あるいは加熱工程等の原理の異なるウイルス除去及び不活化工程が存在しているので、ウイルスクリアランスが期待される。 各製造工程のウイルス除去・不活化効果は、「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（医薬発第 1047 号、平成 11 年 8 月 30 日)」に従い、ウシウイルス性下痢ウイルス（BVDV)、仮性狂犬病ウイルス（PRV)、ブタパルボウイルス（PPV)、A 型肝炎ウイルス（HAV）または脳心筋炎ウイルス（EMCV）をモデルウイルスとして、ウイルスプロセスバリデーションを実施し、評価を行っている。今回報告したリンパ球性脈絡膜髄膜炎ウイルス（LCMV）は、エンベロープの有無、核酸の種類等からモデルウイルスとしては BVDV が該当すると考えられるが、上記バリデーションの結果から、BVDV の除去・不活化効果を有することを確認している。 また、これまでに当該製剤による LCMV 感染の報告例は無い。 以上の点から、当該製剤は LCMV に対する安全性を確保していると考える。

*現在製造を行っていない

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A New Arenavirus in a Cluster of Fatal Transplant-Associated Diseases

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ABSTRACT

BACKGROUND

Three patients who received visceral-organ transplants from a single donor on the same day died of a febrile illness 4 to 6 weeks after transplantation. Culture, polymerase-chain-reaction (PCR) and serologic assays, and oligonucleotide microarray analysis for a wide range of infectious agents were not informative.

METHODS

We evaluated RNA obtained from the liver and kidney transplants in two recipients. Unbiased high-throughput sequencing was used to identify microbial sequences not found by means of other methods. The specificity of sequences for a new candidate pathogen was confirmed by means of culture and by means of PCR, immunohistochemical, and serologic analyses.

RESULTS

High-throughput sequencing yielded 103,632 sequences, of which 14 represented an Old World arenavirus. Additional sequence analysis showed that this new arenavirus was related to lymphocytic choriomeningitis viruses. Specific PCR assays based on a unique sequence confirmed the presence of the virus in the kidneys, liver, blood, and cerebrospinal fluid of the recipients. Immunohistochemical analysis revealed arenavirus antigen in the liver and kidney transplants in the recipients. IgM and IgG antiviral antibodies were detected in the serum of the donor. Seroconversion was evident in serum specimens obtained from one recipient at two time points.

CONCLUSIONS

Unbiased high-throughput sequencing is a powerful tool for the discovery of pathogens. The use of this method during an outbreak of disease facilitated the identification of a new arenavirus transmitted through solid-organ transplantation.

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METHODS OF CLONING NUCLEIC ACIDS of microbial agents directly from clinical specimens offer new opportunities for the surveillance and discovery of pathogens. Molecular techniques have been used successfully in the identification of infectious agents such as the Borna disease virus, hepatitis C virus, Sin Nombre virus, human herpesviruses 6 and 8, *Bartonella henselae*, *Tropheryma whipplei*, West Nile virus, and the coronavirus associated with severe acute respiratory syndrome.[1]

The arenaviruses are enveloped, negative-strand RNA viruses in rodents; these viruses are most frequently transmitted to humans through exposure to infected urine. Infection with the prototype virus, lymphocytic choriomeningitis virus (LCMV), is typically asymptomatic or associated with mild, transient illness; however, LCMV has also been implicated in aseptic meningitis.[2] Human-to-human transmission of LCMV during pregnancy has been reported, and infection during the gestational period can result in fetal death, neurologic sequelae, and chorioretinopathy.[3] Fatal outbreaks of disease associated with human-to-human transmission of LCMV in recipients of solid-organ transplants have also been described.[4] We report the use of unbiased DNA sequencing in the discovery of a new LCMV-related arenavirus that caused fatal disease in three recipients of organs from a single donor.

METHODS

PATIENTS AND CLINICAL COURSE

Three women in Australia who were 63 years of age (Recipient 1), 64 years of age (Recipient 2), and 44 years of age (Recipient 3) received a liver transplant (Recipient 2) or kidney transplants (Recipients 1 and 3) from one male donor who was 57 years of age. The donor died of cerebral hemorrhage 10 days after returning to Australia from a 3-month visit to the former Yugoslavia, where he had traveled in rural areas. The immediate post-transplantation course in the three transplant recipients was unremarkable; however, febrile illnesses with varying degrees of encephalopathy developed in all three, and they died 4 to 6 weeks after transplantation (Table 1). Bacterial and viral cultures; polymerase-chain-reaction (PCR) assays for herpesviruses 1 through 8, lyssavirus, influenza A and B viruses, respiratory syncytial virus, picornavirus, adenovirus, human parainfluenza virus, flavivirus, alphavirus, hantavirus, polyomavirus, Crimean–Congo hemorrhagic fever virus, Rift Valley fever virus, toxoplasma, *Mycobacterium tuberculosis*, and *Mycoplasma pneumoniae*; and viral and panmicrobial oligonucleotide microarray analysis[4] revealed no candidate pathogens.

UNBIASED HIGH-THROUGHPUT SEQUENCING

RNA was extracted from the brain, cerebrospinal fluid, serum, kidney, and liver of Recipient 1, who had received a kidney transplant, and from the cerebrospinal fluid and serum of Recipient 2, who had received a liver transplant. As shown in Figure 1, after digestion with DNase I to eliminate human chromosomal DNA, RNA preparations were amplified by means of reverse-transcriptase PCR (RT-PCR) with the use of random primers.[5,6] Amplification products were pooled and sequenced with the use of the GSL FLX platform (454 Life Sciences), but DNA fragmentation was omitted.[7] After trimming to remove sequences derived from the amplification primer and after filtration to eliminate highly repetitive sequences, the data set was analyzed by subtracting fragments that matched human sequences, clustering nonredundant sequences,[8] and assembling them into contiguous sequences[9] for direct comparison with the GenBank databases of nucleic acids and proteins with the use of BLASTN and BLASTX software.[10] We analyzed the resulting alignments and assigned them to nodes in the National Center for Biotechnology Information taxonomy database, using a custom software application written in Perl (BioPerl version 5.8.5).

PCR QUANTITATION OF THE ARENAVIRUS BURDEN

RNA obtained from tissues, plasma, serum, and cerebrospinal fluid was reverse transcribed with the use of random hexamers. PCR was performed with the use of a SYBR Green assay (Applied Biosystems). The following cycling conditions were used: 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Real-time PCR assays were performed with the following primer set: 5′AGTGCYTGCACAACATCGTTT3′ (forward) and 5′CAATGCCCAGCYTGACAAT3′ (reverse). Thermal cycling was performed with the use of an ABI 7500 real-time PCR system (Applied Biosystems).

Table 1. Characteristics of the Organ-Transplant Recipients.

Recipient No.	Age	Diagnosis	Organ Transplanted	Clinical Course	Interval between Transplantation and Death
	yr				*days*
1	63	End-stage renal failure due to polycystic kidney disease	Kidney	Fever, sepsis, encephalopathy, acute tubular necrosis, graft rejection, radiographic evidence of chest infiltrates	36
2	64	Decompensated cirrhosis and hepatocellular cancer due to hepatitis C infection	Liver	Fever, confusion, encephalopathy with myoclonus, chest infiltrates	30
3	44	End-stage renal failure due to polycystic kidney disease	Kidney	Fever, graft rejection, intraabdominal hematomas and effusion, transplant nephrectomy, encephalopathic illness	29

VIRAL ISOLATION AND ANALYSES

Kidney tissue from Recipient 1 was homogenized in phosphate-buffered saline, centrifuged to pellet cellular debris, filtered, and used to inoculate Vero E6 cells. The cells were monitored daily by means of light microscopy for cytopathic effect and by means of RT-PCR for the presence of arenavirus nucleic acid in supernatant. Monolayers of cells showing cytopathic effects that were also positive for arenavirus nucleic acid were fixed with buffered 4% paraformaldehyde for indirect immunofluorescence and immunohistochemical microscopy and with buffered 2.5% glutaraldehyde for thin-section electron microscopy. Rabbit polyclonal antiserum against Old World arenaviruses, including LCMV, was used as the source of primary antibodies for immunohistochemical analysis. Secondary antibodies were alkaline phosphatase–conjugated goat antibodies against rabbit IgG.[4] Immunohistochemical assays were also performed with the use of formalin-fixed, paraffin-embedded tissue sections obtained from the liver and kidney of Recipient 1.

Virus-infected and noninfected (control) Vero E6 cells were fixed with methanol. Serum specimens from the donor, from the recipients, and from 100 randomly chosen control recipients of solid-organ transplants were applied to the fixed cells followed by fluorescein-labeled antihuman IgG or IgM secondary antibodies.

COMPLETE GENOME SEQUENCING AND PHYLOGENETIC ANALYSES

RNA extracted from the liver in Recipient 1 was used as a template to clone and sequence the L and S segments of the virus. The gene fragments obtained by means of pyrosequencing were used to design specific PCR primers; thereafter, consensus primers were designed on the basis of alignments of other arenavirus sequences with the use of the SCPrimer program.[11]

The L and S segments were assembled and sequenced as a series of overlapping genetic fragments. Evolutionary distances between the assembled segments were computed with the use of the Poisson correction method and expressed in units of amino acid substitutions per site in relationship to arenavirus L, glycoprotein precursor, and nucleoprotein amino acid segments in the GenBank database with the use of the MEGA program.[12] The percentage of replicate trees in which taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (see Fig. 1a, 1b, and 1c of the Supplementary Appendix, available with the full text of this article at www.nejm.org). The nucleotide and amino acid homologies of each of the arenavirus genes (Z, L, GPC, and NP) to LCMV (the closest completely sequenced relative) are shown in Table 2. The sequences are deposited in GenBank (accession numbers EU136038 and EU136039).

RESULTS

RNA from tissue from Recipient 2, who had received a liver, and Recipient 1, who had received a kidney, was pooled and amplified for unbiased high-throughput sequencing,[7] yielding 103,632 se-

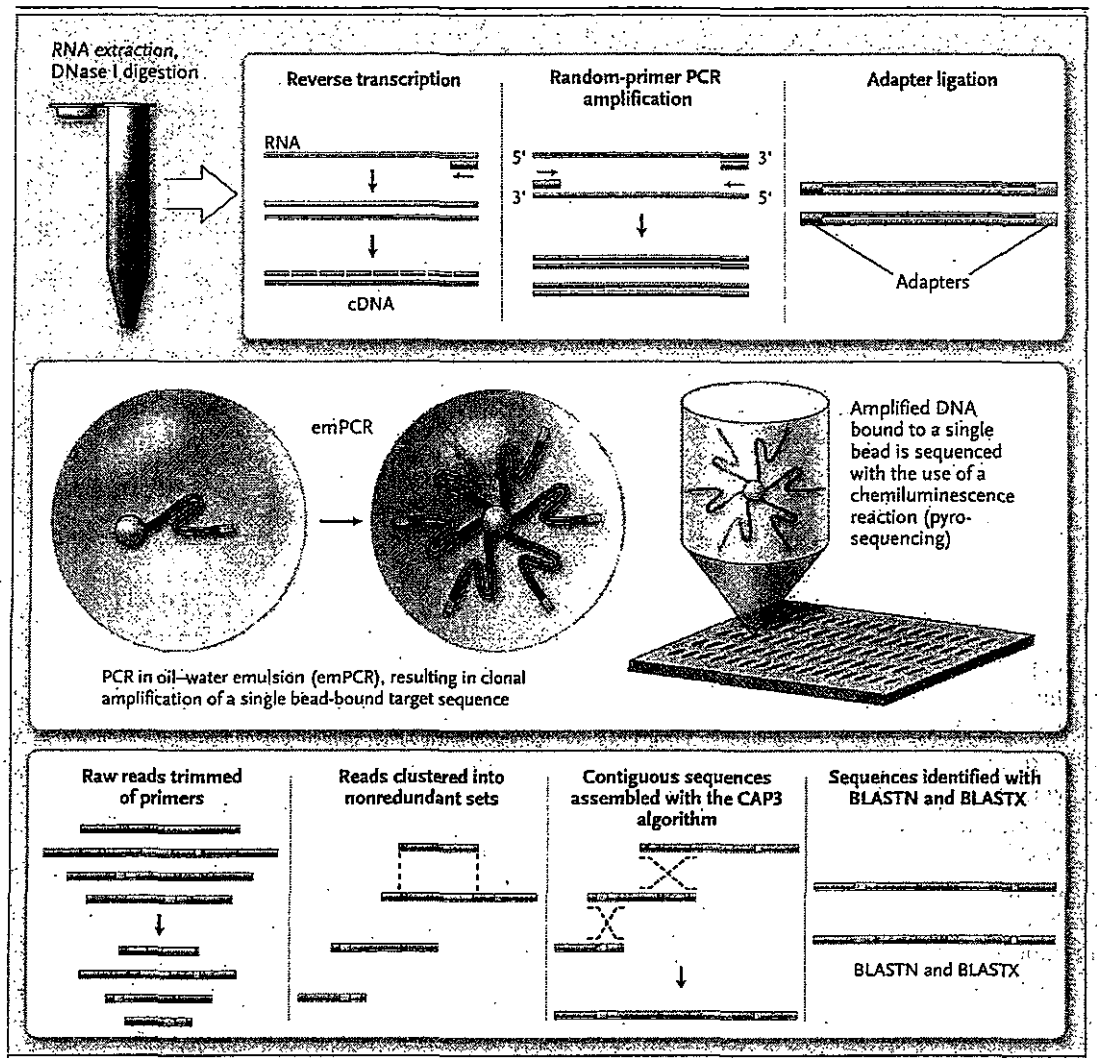

Figure 1. High-Throughput Sequencing Method.

PCR denotes polymerase chain reaction.

quence fragments. The sequences recovered ranged in size from 45 to 337 nucleotides, with a mean length of 162. Sequences derived from the amplification primer and highly repetitive sequences were eliminated, yielding a net of 94,043 sequences. These sequences were processed with the use of algorithms that subtract vertebrate sequences, assemble contiguous sequences, and compare the residual nucleotide and deduced amino acid sequences in all six potential open reading frames with motifs represented in databases of microbes.

At the nucleotide level, sequence data were uninformative; however, BLASTX analysis of the deduced protein sequence revealed 14 fragments that were consistent with Old World arenaviruses (12 S-segment and 2 L-segment fragments) sharing the closest relationship to LCMV.

Primers were designed for RT-PCR experiments to detect viral RNA in clinical specimens, assess the similarity of viral sequences among individual organs and recipients, and extend the viral sequence needed to facilitate characterization. Viral RNA was present in a total of 22 of 30 specimens of tissue, blood, or cerebrospinal fluid from all three transplant recipients (Table 3). The sequence was identical in all specimens, a finding that was consistent with the introduction of a single virus into all the recipients.

Fresh-frozen kidney tissue from Recipient 1 was homogenized and used to inoculate cultures of