別紙3

研究報告調査報告書

識別番号・報告回数				第一報入手日 ：平成22年8月18日	新医薬品等の区分 ：該当なし
一般的名称	－		研究報告の公表状況	－	公表国： 日本
販売名（企業名）	－				

総合機構処理欄

研究報告の概要	インド、パキスタンが発生源とみられ、ほとんどの抗生物質が効かない新種の細菌に感染した患者が欧州などで増えており、ベルギーで16日までに最初とみられる死者が確認された。欧州メディアによると、英国、フランス、ベルギー、オランダ、ドイツ、米国、カナダ、オーストラリアで感染が確認され、今後さらに拡大する恐れがあるという。英国では約50件の感染が確認されている。感染者の多くは、医療費の安いインドやパキスタンで美容整形手術などを受けており、感染源は両国との見方を示している。

使用上の注意記載状況等・その他参考事項等

報告企業の意見	今後の対応
本報告は，当該生物由来製品による感染症情報ではない． 本報告を“新規感染症”および“重大な感染症情報”と考え，報告する．	今後も感染症情報の収集に努め，当該生物由来製品に係る情報を入手した場合には速やかに調査・報告を行い安全性の確保に努める．

MedDRA/J Ver. 13.1J

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サイエンス

細菌：新種の感染が拡大 ベルギーで初の死者

インド、パキスタンが発生源とみられ、ほとんどの抗生物質が効かない新種の細菌に感染した患者が欧州などで増えており、ベルギーで16日までに最初とみられる死者が確認された。欧米メディアによると、英国、フランス、ベルギー、オランダ、ドイツ、米国、カナダ、オーストラリアで感染が確認され、今後さらに拡大する恐れがあるという。

英医学誌ランセット最新号によると、何種類かの細菌が特定の抗生物質を分解する酵素「NDM1」を作り出す遺伝子を持ち、ほとんどすべての抗生物質に対して耐性を持つようになった。こうした細菌に感染すると死亡率が非常に高くなるため、感染への監視強化と新薬の開発が必要だとしている。

同誌によると、英国では約50件の感染が確認されている。感染者の多くは、医療費の安いインドやパキスタンで美容整形手術などを受けており、同誌は感染源は両国との見方を示している。

ベルギー・メディアによると、新たな細菌に感染した男性が死亡したのは今年6月。男性は旅行中にパキスタンで交通事故に遭い、現地の病院で手当てを受けた。ベルギーに戻った時には既に感染しており、投与した抗生物質も効かなかったという。

インド保健省は、感染源をインドと関連づけることに反発し「インドへの医療目的の観光は安全だ」と主張している。（ブリュッセル共同）

毎日新聞　2010年8月17日　19時06分

100

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
			2010. 4. 27	該当なし	
一般的名称	新鮮凍結人血漿	研究報告の公表状況	Byrnes EJ 3rd, Li W, Lewit Y, Ma H, Voelz K, Ren P, Carter DA, Chaturvedi V, Bildfell RJ, May RC, Heitman J. PLoS Pathog. 2010 Apr 22;6(4):e1000850.	公表国 米国	
販売名(企業名)	新鮮凍結血漿「日赤」(日本赤十字社) 新鮮凍結血漿-LR「日赤」(日本赤十字社) 新鮮凍結血漿-LR「日赤」成分採血(日本赤十字社)				

研究報告の概要

○米国北西部に現れた高病原性*Cryptococcus gattii* (*C.gattii*)

*C.gattii*は、免疫抑制状態にある患者のみならず健常人へも感染し、致死性の疾病を引き起こす。この真菌は従来、熱帯・亜熱帯性真菌と考えられていたが、1999年にカナダのバンクーバー島の気温の上昇により流行したと考えられ、隣接するカナダ本土ブリティッシュコロンビアや米国本土にも拡大している。この大流行は*C.gattii type* VGII、特に、VGIIa/majorが原因であったが、新しい遺伝子型(VGIIc型)の*C.gattii*の感染が出現し、数人が死亡している。オレゴンで見つかった新たなVGIIcは免疫細胞あるいはマウスに対して病原性が高いことが示された。新たな気候の中で疾病を引き起こすために、病原体がこれらの地域に適応する方法を確立し、高病原性になっていく可能性を示唆する。

使用上の注意記載状況・その他参考事項等

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

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
米国北西部で見つかり、致死性の疾病を引き起こす高病原性*Cryptococcus gattii* は、従来、熱帯・亜熱帯性真菌と考えられていたが、新しい気候の中でその土地に適応していった可能性があるとの報告である。	日本赤十字社では、輸血感染症対策として問診時に海外渡航歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、発熱などの体調不良者を献血不適としている。今後も引き続き、新興・再興感染症の発生状況等に関する情報の収集に努める。

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PLOS PATHOGENS

Emergence and Pathogenicity of Highly Virulent *Cryptococcus gattii* Genotypes in the Northwest United States

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Abstract

Cryptococcus gattii causes life-threatening disease in otherwise healthy hosts and to a lesser extent in immunocompromised hosts. The highest incidence for this disease is on Vancouver Island, Canada, where an outbreak is expanding into neighboring regions including mainland British Columbia and the United States. This outbreak is caused predominantly by *C. gattii* molecular type VGII, specifically VGIIa/major. In addition, a novel genotype, VGIIc, has emerged in Oregon and is now a major source of illness in the region. Through molecular epidemiology and population analysis of MLST and VNTR markers, we show that the VGIIc group is clonal and hypothesize it arose recently. The VGIIc outbreak lineages are sexually fertile and studies support ongoing recombination in the global VGII population. This illustrates two hallmarks of emerging outbreaks: high clonality and the emergence of novel genotypes via recombination. In macrophage and murine infections, the novel VGIIc genotype and VGIIa/major isolates from the United States are highly virulent compared to similar non-outbreak VGIIa/major-related isolates. Combined MLST-VNTR analysis distinguishes clonal expansion of the VGIIa/major outbreak genotype from related but distinguishable less-virulent genotypes isolated from other geographic regions. Our evidence documents emerging hypervirulent genotypes in the United States that may expand further and provides insight into the possible molecular and geographic origins of the outbreak.

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Introduction

Newly emerging and reemerging diseases have become a major focus of infectious disease research in the 21st century. Reemerging diseases are classified as those that have been previously documented, but are now rapidly increasing in incidence, geographic range, or both [1]. Emerging disease events have been occurring at higher than average rates in the United States due to several factors such as wildlife diversity, environmental change, international travel, and increases in host susceptibility [2,3]. An additional factor contributing to increases in morbidity and mortality for many infectious diseases involves genetic recombination events or gene/pathogenicity island acquisitions. These events can occur via either horizontal gene transfer or conjugation/introgression, leading to novel pathogenic genotypes. This form of virulence evolution has been well characterized in bacterial, viral, fungal, and parasitic human diseases [4,5,6,7,8,9]. The ability to cause damage to mammalian hosts is a common theme among all microbial pathogens, making it a key aspect of host-pathogen studies [10].

In the genomic era, it is now possible to combine conventional epidemiological approaches with newly developed molecular typing techniques to gain insight into the emergence and molecular epidemiology of pathogens. These approaches can improve understanding of population dynamics during an outbreak, and may lead to novel methods for the rapid identification, treatment, and diagnosis of emerging infections [11]. In addition, molecular typing serves as an initial approach to classify isolates into distinct genotypes for analysis. Further investigations may include the examination of virulence and phenotypic traits that may be common or distinct between genotypes [6,12,13]. Gaining insights into the molecular epidemiology and virulence of newly emerging diseases has considerable potential for the rapid assessment and management of newly emerging infections.

Over the past decade, Cryptococcus gattii has emerged as a primary pathogen in northwestern North America, including both Canada and the United States [6,13,14,15,16,17,18]. In the past, C. gattii has often been associated with Eucalyptus trees in tropical and subtropical climates, causing disease in immunocompetent

Author Summary

Emerging and reemerging infectious diseases are increasing worldwide and represent a major public health concern. One class of emerging human and animal diseases is caused by fungi. In this study, we examine the expansion on an outbreak of a fungus, *Cryptococcus gattii*, in the Pacific Northwest of the United States. This fungus has been considered a tropical fungus, but emerged to cause an outbreak in the temperate climes of Vancouver Island in 1999 that is now causing disease in humans and animals in the United States. In this study we applied a method of sequence bar-coding to determine how the isolates causing disease are related to those on Vancouver Island and elsewhere globally. We also expand on the discovery of a new pathogenic strain recently identified only in Oregon and show that it is highly virulent in immune cell and whole animal virulence experiments. These studies extend our understanding of how diseases emerge in new climates and how they adapt to these regions to cause disease. Our findings suggest further expansion into neighboring regions is likely to occur and aim to increase disease awareness in the region.

hosts at low incidences [19,20,21]. *C. gattii* is distinct from its sibling species *Cryptococcus neoformans* [22], which more commonly infects immunosuppressed hosts and infects almost one million people annually with over 620,000 attributable mortalities [23,24,25]. *C. gattii* can be classified into four discrete molecular types (VGI-VGIV), which represent cryptic species as no nuclear allelic exchange between groups has been observed [6]. This molecular classification is significant because VGII is responsible for approximately 95% of the Pacific Northwest infections in Canada and the United States [12,15]. The appearance of *C. gattii* in North America is alarming because this is the first major emergence in a temperate climate, indicating a possible expansion in the endemic ecology of this pathogen [26,27].

Several significant questions persist regarding the outbreak and its expansion within the United States. As the global collection of *C. gattii* isolates expands, the molecular epidemiology of the species has become increasingly informative, particularly through multilocus sequence typing (MLST), which allows data to be readily compared between groups within the research community [6,15,28,29,30]. The increase in global and regional isolates that have been typed at the molecular level allows detailed analysis of *C. gattii*. The analysis of both conserved coding regions, and diverse noncoding regions provides insight into the genotypes responsible for the outbreak. A major finding in this study is a level of underlying diversity within the VGIIa/major genotype in the region of expansion and other geographic locales.

Prior studies documented that the *C. gattii* VGIIa/major genotype isolates from Vancouver Island are highly virulent in experimental murine infection assays [6]. Here we expanded this analysis to examine clinical VGIIa genotype isolates from Vancouver Island, the United States, and Brazil, in addition to an environmental VGIIa isolate from California. Our findings are consistent with recent macrophage intracellular proliferation studies, demonstrating that United States isolates from the recent Pacific NW outbreak exhibit high virulence [31]. The enhanced virulence of isolates from the outbreak region, when compared with those from other regions, suggests that the genotypes circulating in the Pacific NW are inherently increased in their predilection to cause disease in mammalian hosts.

In addition to the detailed examination of the VGIIa/major genotype clade, we report that the novel VGIIc genotype is highly virulent in a murine inhalation model. Moreover, the VGIIc genotype was found to have high intracellular proliferation rates in macrophages and a significantly increased percentage of mitochondria with tubular morphology after macrophage exposure, and thus VGIIc isolates share virulence attributes with the VGIIa/major genotype isolates from the Vancouver Island outbreak. These results extend the molecular and phenotypic understanding of the recently discovered VGIIc/novel genotype and help shed light into its possible geographic and molecular origins.

These studies provide insights into both the evolutionary history and virulence characteristics of this unique and increasingly fatal fungal outbreak in the temperate climate of the North American Pacific Northwest and highlight the importance of a collaborative interdisciplinary approach to the analysis of emerging pathogens. Application of these approaches may increase awareness of disease risks in the expansion zone, lead to more rapid diagnoses and, as a result, accelerate the implementation of appropriate therapy.

Materials and Methods

Isolate Identification

Human and veterinary cases of confirmed or suspected *C. gattii* infections in the states of Washington and Oregon were identified by referring physicians and veterinarians, and subsequently isolates were purified and examined. Melanin production was assayed by growth and dark pigmentation on Staib's niger seed medium, and urease activity was detected by growth and alkaline pH change on Christensen's agar. These tests established that isolates were *Cryptococcus* (*C. neoformans* or *C. gattii*). Isolates were concomitantly examined for resistance to canavanine and utilization of glycine on L-canavanine, glycine, 2-bromothymol blue (CGB) agar. Growth on CGB agar indicates that isolates are canavanine resistant, and able to use glycine as a sole carbon source, triggering a bromothymol blue color reaction indicative of *C. gattii*, whereas *C. neoformans* is sensitive to canavanine, and cannot use glycine as a sole carbon source, resulting in no growth or coloration in this selective indicator medium. All CGB positive isolates were then grown under rich culture conditions prior to storage at −80°C in 25% glycerol and genomic DNA extraction. For genomic DNA isolation, a modified protocol of the MasterPure Yeast DNA purification kit from Epicentre Biotechnologies was used. Briefly, 500 µl of glass beads (425–600 nm) were added into the combination of cells and 300 µl cell lysis solution. The rest of the method followed the protocol provided by the manufacturer.

Molecular Epidemiology

For multilocus sequence typing analysis (MLST) [32], each isolate was analyzed with a minimum of eight and in some cases sixteen loci. For each isolate, genomic regions were PCR amplified (Table S1), purified (ExoSAP-IT), and sequenced. All primers used for the analysis were designed specifically to amplify open reading frame (ORF) gene sequence regions including those with non-coding DNA regions to maximize discriminatory power. Sequences from both forward and reverse strands were assembled, and manually edited using Sequencher version 4.8 (Gene Codes Corporations). Based on BLAST analysis of the GenBank database (NCBI), each allele was assigned a corresponding number. GenBank accession numbers with corresponding allele numbers are listed in the supplementary information (Table S2). To determine that the nine VGIIc/novel isolates are clonally related, given the level of diversity in the loci and the number of isolates that have been examined, we applied an equation to measure the probability of a genotype occurring more than once in the dataset [33,34]. For the variable number of tandem repeat (VNTR) analysis, the Tandem Repeat Finder (TRF) version 4.00 software package was employed for marker development, using the genomic sequence of *C. gattii* isolate R265 (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b.2/Home.html) [35]. The identified tandem repeat sequences and 400 bp of the flanking region were extracted from the genomic sequence and ranked according to the number of total repeats and the size of repeat units using an in-house Perl script (available upon request). Markers were examined for stability and those with high variability and stability were chosen for the analysis. Sequences were assembled and edited using Sequencher version 4.8 (Gene Codes Corporations) and aligned using the Clustal W web based software package (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Mating Conditions

Mating analysis was conducted on V8 media (pH 5). Isolates were incubated at room temperature in the dark for 2–4 weeks in dry conditions. All strains were crossed with the VGIII mating type a isolate B4546 and the VGIII mating type α isolate NIH312, both of which are fertile and commonly used for mating studies [36]. Fertility was assessed by microscopic examination for hyphae, fused clamp cells, basidia, and basidiospore formation.

Clustering and Haplotype Analyses

For each VNTR marker, a sequence type was defined as a sequence exhibiting a unique mutation. Each sequence type was confirmed to be unique by BLAST analysis of the NCBI GenBank database [37]. A concatenated VNTR sequence type (CVST) was defined as unique combinations of sequence types from the VNTR markers. A multiple alignment of the sequences was carried out using Clustal W software [38]. Analysis of the sequences was conducted using the Neighbor-Joining and Maximum Parsimony methods within the MEGA 3.1 software [39]. In addition, the use of the maximum likelihood method (PhyML 3.0) with SH-like approximate likelihood-ratio test and HKY85 substitution model was applied [40,41]. For this purpose, sequences of the selected VNTR markers were concatenated. We additionally concatenated all of the strain-typing markers including the housekeeping genes used in MLST and VNTR loci for clustering analysis. The haplotype mapping analysis was carried out using TCS software version 1.21 (http://darwin.uvigo.es/software/tcs.html) [42].

Intracellular Proliferation Rate (IPR) Determination

A proliferation assay was previously developed to monitor the intracellular proliferation rate (IPR) of individual strains for a 64-hour period following phagocytosis [31]. For this assay, J774 macrophage cells were exposed to cryptococcal cells that were opsonized with 18B7 antibody for 2 hr as described previously [43]. Each well was washed with phosphate-buffered saline (PBS) in quadruplicate to remove as many extracellular yeast cells as possible and 1 ml of fresh serum-free DMEM was then added. For time point T = 0, the 1 ml of DMEM was discarded and 200 µl of sterile dH_2O was added into wells to lyse macrophage cells. After 30 minutes, the intracellular yeast were released and collected. Another 200 µl dH_2O was added to each well to collect the remaining yeast cells. The intracellular yeast were then mixed with Trypan Blue at a 1:1 ratio and the live yeast cells were counted. For the subsequent five time points (T = 16 hrs, T = 24 hrs, T = 40 hrs, T = 48 hrs and T = 64 hrs), intracellular cryptococcal cells were collected and independently counted with a hemocytometer. For each strain tested, the time course was repeated at least three independent times, using different batches of macrophages. The IPR value was calculated by dividing the maximum intracellular yeast number by the initial intracellular yeast number at T = 0. We confirmed that Trypan Blue stains 100% of the cryptococcal cells in a heat-killed culture, but only approximately 5% of cells from a standard overnight culture. Compared to a conventional colony counting method, this method was shown to be more sensitive in detecting the clustered yeast population or yeast cells undergoing budding. IPR values were used to assess how consistent the different VGII genotype subgroups were. For this statistical analysis the medians of each population were compared with the non-parametric Mann-Whitney U-test and values of $p<0.025$, after controlling for multiplicity, and were accepted as statistically significant (http://elegans.swmed.edu/~leon/stats/utest.cgi).

Mitochondrial Morphology

The mitochondrial morphology assays were conducted in a similar way to those in previous studies, with modifications [31]. *C. gattii* cells, grown overnight at 37°C in DMEM in a 5% CO_2 incubator without shaking for 24 hr, or isolated from macrophages 24 hr after infection, were harvested, washed with PBS twice and re-suspended in PBS containing the Mito-Tracker Red CMXRos (Invitrogen) at a final concentration of 20 nM. Cells were incubated for 15 min at 37°C. After staining, cells were washed in triplicate and re-suspended in PBS. For each condition, more than 100 yeast cells per replicate for each of the tested strains were chosen randomly and analyzed. For quantifying different mitochondrial morphologies, images were collected using a Zeiss Axiovert 135 TV microscope with a 100× oil immersion Plan-Neofluar objective. Both fluorescence images and phase contrast images were collected simultaneously. Images were captured with identical settings on a QIcam Fast 1394 camera using the QCapture Pro51 version 5.1.1 software. All Images were processed identically in ImageJ and mitochondrial morphologies were analyzed and counted blindly.

Three individual experiments were performed for each condition and the data were tested for normality using the Shapiro-Wilk test. For homogeneity of variances we used the Levene statistic. For statistically significant differences among the mean data we applied a One-Way ANOVA. Multi-comparisons using Tukey Honestly Significant Differences tests were performed to identify statistically significant differences between pairs. A p-value of $p<0.05$, after controlling for multiplicity, was considered to be statistically significant. Regression analysis was used to measure the correlation between tubular mitochondrial morphology and IPR values; an F-value of $P<0.05$ was considered to be a significant correlation.

Murine Virulence Tests and Histopathology

To examine the virulence potential of global VGII isolates, with a specific emphasis on the Pacific NW VGII outbreak genotypes, two independent murine virulence experiments were conducted at two facilities (Duke University Medical Center and the Wadsworth Center). The murine virulence assays at Duke University Medical Center and the Wadsworth Center used a similar protocol to previous *C. gattii* and *C. neoformans* experimental infections [6,44,45].

At the Duke University Medical Center Animal Facility, virulence was assessed using female A/Jcr mice (NCI, 18–24 g). Strains were cultured in YPD broth for 18–20 h at 30°C, harvested, washed three times with sterile PBS and counted using a hemocytometer to determine cell concentrations. Inocula for both murine experiments were confirmed by plating on YPD and counting colony-forming units (c.f.u.). Nine to ten A/Jcr mice per strain were anesthetized with pentobarbital and infected via intranasal instillation with 5×10^4 c.f.u. in 50 µl of sterile 1× PBS.

Animals that displayed severe morbidity, based on twice-daily examinations, were euthanized. Time to mortality was evaluated for statistical significance using Kaplan–Meier survival curves within the Prism software package (GraphPad Software), and P values were obtained from a log-rank test. Survival data was plotted for graphical analysis using the Prism software package.

At the Wadsworth center animal facility, all assays were conducted using male BALB/c mice (approximately 6 weeks old, 15–20 g, Charles River Laboratories, Inc.). Strains were grown overnight in YPD broth at 30°C with shaking. The cells were harvested, washed in PBS, and counted using a hemocytometer. Five mice per strain were anesthetized with a mixture of xylazine–ketamine, and allowed to inhale 10^5 (30 µl) cryptococcal cells per mouse, via intranasal instillation. Mice were given food and water *ad libitum* and monitored twice daily. At the first sign of poor health or discomfort, infected animals were euthanized. Brain and lung tissues from the dead animals were cultured on Niger seed agar for *C. gattii* recovery to confirm infections were due to this pathogen. Time to mortality was evaluated for statistical significance as described above.

Two animals from each strain assayed in the study conducted at Duke University were selected for histopathology analysis either at the time of sacrifice or at the conclusion of the experiment for the more attenuated isolates. For each animal, lung samples were collected and stored in 10% neutral buffered formalin. Samples were paraffin embedded and hematoxylin and eosin (H&E) stained at the Duke University Research Histology Laboratory. After staining and slide preparation, each sample was examined microscopically for analysis of cryptococcal cell burden and immune responses. Images were captured using an Olympus Vanox microscope (Duke PhotoPath, Duke University Medical Center).

Ethics Statement

The animal studies conducted at the Wadsworth Center were in full compliance with all of the guidelines set forth by the Wadsworth Center Institutional Animal Care and Use Committee (IACUC) and in full compliance with the United States Animal Welfare Act (Public Law 98–198). The Wadsworth Center IACUC approved all of the vertebrate studies. The studies were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

The animal studies at Duke University Medical Center were in full compliance with all of the guidelines of the Duke University Medical Center Institutional Animal Care and Use Committee (IACUC) and in full compliance with the United States Animal Welfare Act (Public Law 98–198). The Duke University Medical Center IACUC approved all of the vertebrate studies. The studies were conducted in Division of Laboratory Animal Resources (DLAR) facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Results

Molecular Analysis of *C. gattii* VGII Outbreak vs. Global Isolates

To examine the *C. gattii* outbreak isolates collected from 2005 to 2009 (Figure 1), an in-depth stepwise molecular analysis was applied to each isolate, and the genotypes were compared with other global genotypes. In total, 20 markers were selected for analysis. These markers include both coding and noncoding genomic regions and range in size and allelic diversity (Table 1). Additionally, all of the markers are randomly distributed among the chromosomes in the most recent assembly of the reference *C. gattii* VGI genome, WM276 (Figure 2). Initially, all isolates were sequenced at a total of eight MLST markers, and four variable number of tandem repeat (VNTR) markers (Figure 3, Table 2). Next, global isolates were selected for diversity, and several isolates from each of the primary genotypes in the expansion region were chosen for sequence analysis at eight additional MLST loci, bringing the total number of genetic markers analyzed for these isolates to 20 (Figure 4A). As expected, the MLST markers were less variable and more conserved, while the *VNTR* markers allowed for higher-resolution differentiation between isolates that appeared identical by MLST analysis. The generated datasets were then concatenated both without and with VNTR data (Figure 4B, Figure 4C).

The combined analysis of the results presented here, and a 30 marker MLST analysis conducted previously [6,18], reveal several findings of interest in relation to VGII genotypes in the region. From the analysis of 34 markers (30 MLST/4 VNTR), we show that the Vancouver Island VGIIa/major isolates are fully identical at all loci to several recent isolates from Washington and Oregon, as well as a historical clinical isolate (1970's), NIH444, from Seattle. Additionally, the VGIIb/minor isolates from Australia and Vancouver Island are identical at 34 total loci, and also identical to VGIIb/minor isolates from Oregon at 20 loci (16 MLST/4 VNTR). Furthermore, all VGIIc isolates to date are identical across all 20 loci examined (Figure 4A). However, we also are able to discriminate the outbreak VGIIa genotype from an environmental VGIIa isolate from California, CBS7750, and clinical VGIIa isolates CA1014 and ICB107 from California and Brazil, respectively, at one or more MLST/VNTR loci. It is clear from prior studies that the VGIIa/major and VGIIb/minor isolates are clonal lineages [6,12,15,46], and here we confirmed that this is the case for the nine VGIIc/novel isolates, based on 7-loci MLST analysis of the global VGII population (Figure S1) ($p<0.0001$).

The largest and most comprehensive dataset arose from the combined analysis of seven MLST and four VNTR loci, resulting in a total of 41 sequence types (STs). This dataset was generated from clinical, veterinary, and environmental *C. gattii* isolates (Figure 3, Figure S1, Table S3). From the analysis, it is clear that the *VGIIa/b/c clusters are all related to each other*, but also distinct. In addition, the data show that the VGIIa/major clade is closely clustered to VGIIc, further validating prior reports that examined a more limited number of loci [13,47]. In addition, VGIIc (ST21) shares high sequence identity to ST34, represented by a mating type a clinical isolate from Colombia, suggesting that the VGIIc genotype may have resulted from a-α mating, even though all isolates related to the Pacific NW outbreak are exclusively α mating type. Additionally, Vancouver Island isolates from our collection that had not been fully typed by MLST were sequenced at two loci to determine if any were unrecognized VGIIc isolates (n = 56) (Figure S2). Of these, 51 were found to be VGIIa, five were VGIIb, and none were VGIIc, consistent with previous data from the region. Thus, VGIIc appears to remain exclusive to the United States, specifically Oregon, and has never been reported from Vancouver Island, the mainland of Canada, Washington State, or elsewhere globally.

Within the VGIIa/major cluster, based on the initial MLST analysis of 30 loci, only a single isolate (ICB107) could be distinguished from the other VGIIa isolates, and this was at only one locus [18]. To further investigate this homogeneous population causing the vast majority of the outbreak-related morbidity and mortality, we expanded the molecular analysis to include highly variable regions of the genome. The application of these VNTR markers, in combination with the MLST markers,

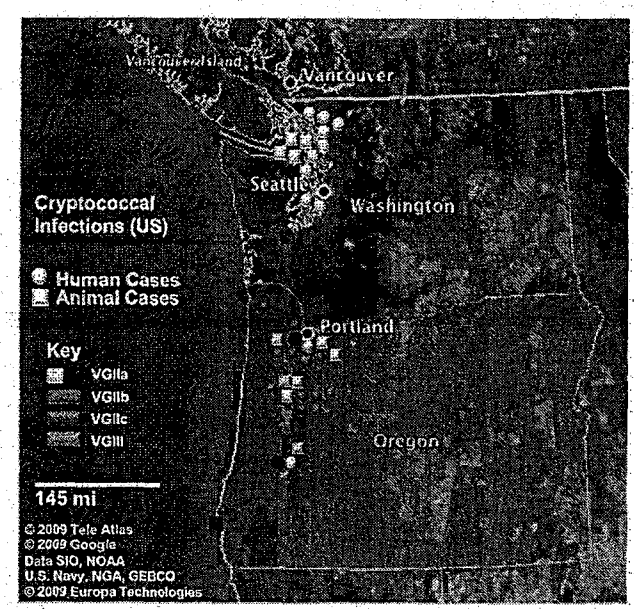

Figure 1. Geographic dispersal of pathogenic *C. gattii* genotypes in the United States. Circles represent human cases and squares represent animal (non-human mammalian) cases. All cases shown have been reported from 2005 to 2009. Isolates are color coded by genotype, in which yellow and blue correspond to VGIIa/major genotype cases (yellow ST1, blue ST30), red corresponds to VGIIb/minor, green corresponds to the novel VGIIc genotype, and orange corresponds to two cases determined to be molecular type VGIII. In total, there were 39 cases (18 human, 21 animal) that have been confirmed by phenotypic and genotypic profiling.
doi:10.1371/journal.ppat.1000850.g001

allowed us to generate five independent STs from within the VGIIa/major genotype and related isolates (Figure 3).

These five sequence types (ST1, ST2, ST3, ST13, ST30) contained a total of 44 isolates (Figure 3, Table S3). The canonical VGIIa/major outbreak genotype, ST1, contained the vast majority of the 44 isolates (n = 38). As expected based on previous models of the *C. gattii* outbreak expansion [13], ST1 consisted of isolates exclusively from the initial outbreak and expansion zones, including British Columbia, Washington, and Oregon (Table S3). These results further validate the hypothesis that the epicenter of the outbreak was on Vancouver Island, beginning in the late 1990's, with a direct expansion into neighboring mainland British Columbia and subsequently into the United States [13]. The only exception in this dataset is isolate NIH444, an older isolate from the region that was isolated from a patient sputum sample in Seattle in the early 1970's [18], which is also identical at all 34 markers examined. This suggests that the VGIIa/major genotype responsible for most of the outbreak cases may have been circulating in the region prior to the outbreak. The possible travel history of this patient is unknown, and could therefore have involved exposure on Vancouver Island. Overall, this analysis provides increased evidence that the outbreak genotype is unique to the region thus far, and molecularly distinct from closely related isolates from both California and South America.

While the homogeneous nature of the VGIIa/major isolates based on robust molecular typing validated previous models, an underlying diversity within this group was also discovered. First, we further validated that the isolate ICB107 (ST13), from Brazil, was indeed distinct from the ST1 VGIIa/major clade. This isolate differs at one MLST marker (*LACI*), and three VNTR markers (VNTR3, VNTR15, VNTR34). Additionally, the high-resolution sequence analysis was able to discriminate other VGIIa isolates that were collected from California. These include isolate CBS7750 (ST3), collected from the environment in San Francisco in 1990 [48], and isolate CA1014 (ST2), which was isolated from a patient with HIV infection in southern California. Each of these two isolates differs from ST1 due to unique mutations within the VNTR7 and VNTR34 loci, respectively. This shows that similar VGIIa genotype isolates have been found elsewhere, but that none are identical to those circulating as part of the ongoing Vancouver Island outbreak. Whether these isolates are a result of drift from ST1, or if ST1 arose from one of these related genotypes is not known.

In addition to discriminating VGIIa isolates that were not from the outbreak region, we also found a novel ST, ST30, which is highly similar to ST1, but divergent at a unique region of VNTR34. Interestingly, all three of the ST30 isolates are exclusively from Oregon, including two human clinical cases and one marine mammal case (Figure 1, Figure 3, Table S3).

Table 1. Markers used in this study.

Marker	Length (bp)	Chromosome (WM276)	Alleles
SXI1α	1,354	9	10
SXI2a	2,529	N/A*	2
IGS	740	2	15
TEF1	700	13	5
GPD1	547	6	10
LAC1	554	7	5
CAP10	569	11	4
PLB1	600	13	9
MPD1	677	7	1
HOG1	564	3	11
BWC1	587	4	7
CNB1	571	10	6
TOR1	574	6	5
CRG1	575	2	9
FHB1	535	3	5
FTR1	545	3	7
CAP59	557	1	9
VNTR3	334	1	13
VNTR7	270	4	21
VNTR15	364	6	19
VNTR34	526	7	32

* *SXI2a* is an idiomorphic allele, and therefore not present in the α mating type isolate WM276.

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These results are consistent with an expansion followed by genetic drift in the highly variable VNTR loci. Isolates of ST30 have not been detected on Vancouver Island, indicating that this divergence is recent, and likely occurred after the expansion of ST1 into the United States. Alternatively, both ST1 (VGIIa/major) and ST30 may have been present for a long period, with only ST1 having been transferred to Vancouver Island.

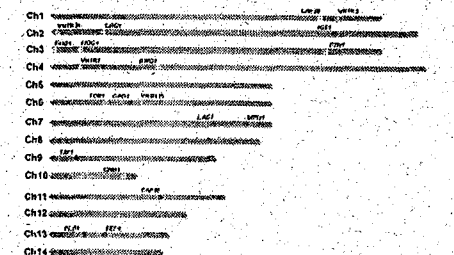

Figure 2. Markers used in the study are dispersed in the genome. A map of each chromosome is represented, illustrating the locations of each marker based on the genomic sequence of the *C. gattii* isolate WM276. MLST markers (n = 16) are indicated on the map by hexagons, with pink denoting the standard set used, blue the expanded set of loci, and red the *MAT* linked locus that is specific to α isolates. Green triangles represent the four VNTR loci that were examined.

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To gain insights into the potential origins of the VGIIc genotype, and to assess its position within the overall VGII clade, clustering analysis was applied. Analysis of the combined dataset including 41 sequence types generated from 115 *C. gattii* isolates shows that the VGIIc genotype is independent, but similar to VGIIa (Figure 3). The closest relationship determined from the analysis was to ST34, an isolate from Colombia, which is also of the opposite a mating type. Moving beyond the direct branch, it appears that the VGIIc genotype shares sequence similarities to global isolates from South America, Africa, and also European isolates with likely African origins based on collected clinical case histories. Additionally, the VGIIc group also shares the *IGS1* allele with isolates from Australia, further obscuring the possible origins and necessitating a more thorough analysis (Figure 4A).

When the clustering analysis was expanded to include additional MLST loci (Figure 4A), both with and without the VNTR markers, the relationships of VGIIc to other global genotypes was further elucidated, with close relationships observed with global isolates from South America, Africa, Europe (Greece), and Australia (Figure 4B, Figure 4C, Table S4). These results increase the comprehensiveness of the analysis, and allow predictions of the relationship of this genotype to global isolates. Examination of alleles illustrates that, when the analysis is expanded, the VGIIc group appears to be more diverse from VGIIa and VGIIb. Each allele represented in green was initially denoted as an allele that was unique to the VGIIc genotype, with a total of seven such alleles (Figure 4A). To further elucidate the possible origins of these alleles, isolates selected based on their global diversity were sequenced at these loci (Figure 4A). Identical matches for four of the seven VGIIc-unique alleles were identified in isolates from Brazil, Australia, Europe, and European isolates with likely African origins, while three alleles (*SXI1α*, *HOG1*, and *CRG1*) remain unique to this novel genotype and only seen in Oregon thus far (Figure 4A).

To further characterize the genetic relationships among the global isolates in relation to the outbreak isolates, maximum likelihood (ML) analysis was applied. Initially, the isolates were characterized at 15 MLST loci, excluding the *MAT* locus so that both α and a isolates could be included. This analysis indicates that VGIIc may be more distantly related to the VGIIa/major genotype than initially observed. In addition, analysis of the 15 MLST loci shows a possible relation of VGIIc with isolates from South America, Africa, Europe, and Australia (Figure 4B). When this analysis was expanded to also include the four VNTR loci, similar results for the global comparisons of all genotypes and the relation of VGIIc to global isolates were observed (Figure 4C). For these reasons, additional sampling and analysis will be necessary to more precisely elucidate if this novel virulent genotype originated locally, or originated in an under-sampled region.

In addition to clustering analyses, TCS haplotype-mapping software was applied to establish the evolutionary histories of the MLST alleles examined during the analysis (Figure 5, Figure 6, Figure S3). From the sequence results, all of the VGIIc isolates were determined to be 100% identical, indicating that there was likely a recent emergence in which all of the isolates are clonally derived. To test this hypothesis, the TCS analysis allowed for the examination of individual loci to determine which alleles are likely ancestral, intermediate, or recently derived. Of the sixteen loci examined, eight were consistent with VGIIc possessing the ancestral allele, six of the alleles were distal nodes at the terminal end of the respective haplotype networks, and two loci were of intermediate allele positions.

Alleles with ancestral genotypes are less informative because these alleles may not have diversified over time in the VGIIc

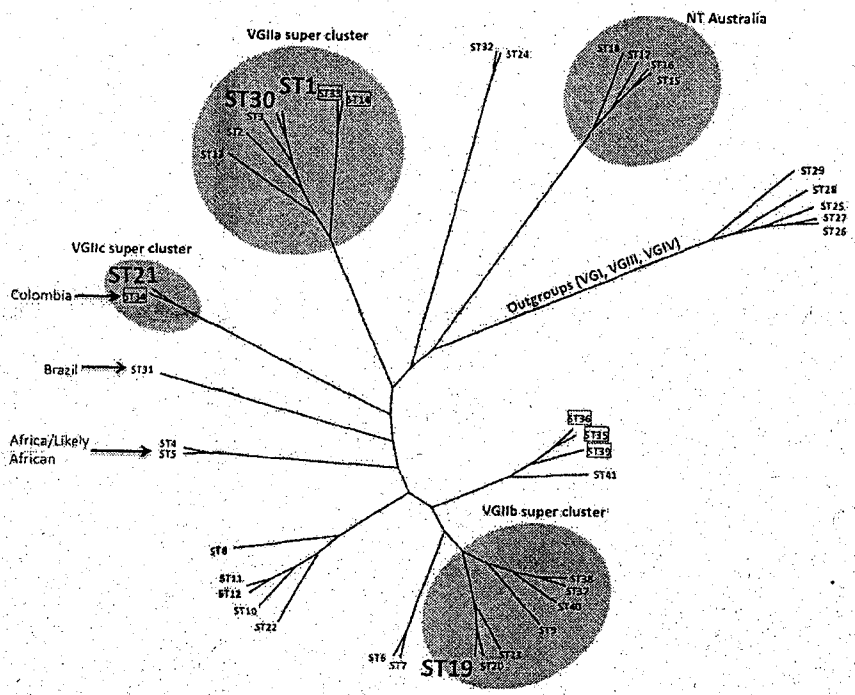

Figure 3. Clustering analysis of global VGII isolates shows high global diversity. This dendrogram, based on seven MLST loci and four VNTR loci, illustrates the global divergence seen in this molecular type. Major clusters are highlighted accordingly to illustrate the placements of the VGIIa/b/c super clusters as well as a unique NT cluster that has been found only in Australia thus far. Sequence types 1, 30, 19, and 20 are enlarged and represent the primary genotypes responsible for the Pacific NW outbreak. Boxed isolates represent those of the a mating type and all other sequence types represent the genotypes observed for mating type α isolates. Several genotypes are also combined with geographic information to illustrate the diversity surrounding several sequence types. Isolates from the VGI, VGIII, and VGIV molecular types serve as out-group sequence types.

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lineage for various reasons, including selection pressures and overall lack of diversity at the allele. When only non-ancestral alleles were examined, 75% lay at the distal ends of their haplotype maps. Intriguingly, the three VGIIc alleles unique to the genotype (*SXI1α*, *HOG1*, and *CRG1*) all have distal placements (Figure 5A–C). Additionally, the most recent ancestor to VGIIc in all three cases can be shown to derive from isolates that are from South America and Australia, indicating that VGIIc may have emerged out of one of these regions (Figure 5). While other regions including Europe and North America can be seen, no other regions are observed for all three of these alleles. These distal placements are consistent with a recent divergence of the unique VGIIc lineage. The haplotype analysis, in combination with the lack of any underlying diversity within the nine VGIIc isolates analyzed, indicates a recent emergence of this novel virulent genotype in Oregon.

To examine the role that recombination may have played in the population structure of the VGII molecular type, we conducted paired allele analysis for 25 representative global isolates (Figure 6, Figure S4). The discovery of all four possible allele combinations between two unlinked loci (AB, ab, Ab, aB) serves as evidence for likely recombination [49]. From this analysis, we show that isolates collected from South America, Africa, and Australia appear to be involved in recombination events. Representative VGIIa/major, VGIIb/minor, and VGIIc/novel isolates were found among groups of recombinant isolates. A group of ten isolates, all α, from South America and Africa (Figure S4) appeared most commonly as recombinant partners, although several a mating type isolates were also less frequently involved. In further support, when we examined the number of genotypes present by region and compared this data to the total number of genotypes represented (Figure S1), it is clear that South America and Africa populations are more diverse when compared with isolates from North America, which are more clonal. Additionally, while the observed diversity in Australia was lower than South America and Africa, this may be attributable to sampling bias of clonal regions as prior studies have shown that this continent is a region with high levels of recombination due to both same-sex and opposite-sex mating

Table 2. Isolates collected from cases within the United States, 2005–2009 (n = 40).

Isolate	Host	Residence	Molecular Type*
T67707	Human	Washington	VGIIa/major
W1S209	Human	Washington	VGIIa/major
EJB4	Human	Washington	VGIIa/major
EJB5	Human	Washington	VGIIa/major
EJB6	Human	Washington	VGIIa/major
EJB7	Human	Washington	VGIIa/major
EJB8	Human	Washington	VGIIa/major
EJB9	Human	Washington	VGIIa/major
EJB13	Human	Washington	VGIIa/major
KB11632	Human	Oregon	VGIIa/major
EJB3	Human	Oregon	VGIIa/major
EJB19	Human	Oregon	VGIIa/major
MMC08-1042	Human	Oregon	VGIIa/major
EJB16	Alpaca	Oregon	VGIIa/major
EJB17	Dog	Oregon	VGIIa/major
3700 (1)	Porpoise	Washington	VGIIa/major
3700 (2)	Porpoise	Washington	VGIIa/major
3635	Porpoise	Washington	VGIIa/major
3059	Porpoise	Washington	VGIIa/major
EJB21	Porpoise	Oregon	VGIIa/major
EJB22	Dog	Oregon	VGIIa/major
EJB51	Alpaca	Oregon	VGIIa/major
EJB54	Cat	Oregon	VGIIa/major
EJB77	Dog	Oregon	VGIIa/major
EJB79	Alpaca	Oregon	VGIIa/major
A6MR38	Human	Oregon	VGIIc/novel
EJB12	Human	Oregon	VGIIc/novel
EJB18	Human	Oregon	VGIIc/novel
EJB14	Cat	Oregon	VGIIc/novel
EJB15	Alpaca	Oregon	VGIIc/novel
EJB52	Cat	Oregon	VGIIc/novel
EJB55	Ovine	Oregon	VGIIc/novel
EJB74	Cat	Oregon	VGIIc/novel
EJB75	Dog	Oregon	VGIIc/novel
EJB10	Human	Oregon	VGIIb/minor
MMC08-896	Dog	Oregon	VGIIb/minor
EJB53	Elk	Oregon	VGIIb/minor
EJB76	Cat	Oregon	VGIIb/minor
EJB11	Human	Washington	VGIII
MMC08-897	Cat	Oregon	VGIII

* The Molecular type designation is based on 8-loci MLST analysis.

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events [50]. In addition to the paired allele analysis, allele diagrams were constructed to observe possible recombination within individual MLST loci (Figure S5). The most parsimonious explanation for allelic diversity in 11 of the MLST loci analyzed is as a result of consecutive and/or independent mutations within the population. Within the four remaining loci, there exists at least one hybrid allele that may be the result of a recombination event between two hypothesized parental alleles in the global VGII population (Table 3, Figure S5). Phenotypic mating results were conducted and illustrate that the VGIIa/major (α), VGIIc/novel (α), VGII mating type a genotypes, as well as several of the proposed parental contributors from the allelic and genotypic recombination analysis show fertility with the production of spores when mated with fertile VGIII isolates (Table S5). Taken together, this suggests that both α-α and a-α mating events may be contributing to the formation of recombinant genotypes as well as the production of infectious spores. There were no examples of alleles introgressed into VGII from VGI, VGIII, or VGIV, in accord with findings that the four VG molecular types likely represent cryptic species [6,29]. In summary, these results suggest that recombination events may be critical driving forces in the evolution of *C. gattii* VGII diversity, which may in part contribute to the generation of genotypes displaying increased virulence.

VGIIc/novel and VGIIa/major Outbreak Isolates Are Hypervirulent

It has recently been shown that intracellular proliferation rate (IPR) values for cryptococcal cells within macrophages are positively correlated with virulence in the murine model for cryptococcosis [31]. To further elucidate the potential virulence of outbreak isolates collected from the United States, proliferation rates of selected isolates were tested and compared to other isolates for which proliferation data had been previously obtained. In total, IPR values for eight of the nine VGIIc isolates were measured (Figure 7A). In addition, the type strains for VGIIa/major (R265) and VGIIb/minor (R272) were included as controls, and previously published data for other VGIIa and VGIIb isolates were included for comparisons [31]. On the basis of individual strains, seven of the eight VGIIc/novel isolates showed high IPR levels, with only a single outlier (EJB52) that had a low IPR value (0.97). Taken together, the median IPR value for VGIIc is significantly closer to that of VGIIa/major than to VGIIb/minor (Figure 7A). These results indicate that the VGIIc genotype has a similar intracellular phenotype, and thus virulence profile to the VGIIa/major genotype. This is noteworthy because previous analysis showed that the VGIIa/major genotype isolates from the outbreak had unusually high IPR values, and the VGIIc isolates from the same outbreak are here shown to have similarly high IPR values.

Another unique feature of the outbreak VGIIa/major isolates is the ability to form highly tubular mitochondria after intracellular parasitism, a characteristic that correlates with both IPR and murine virulence [31]. To explore the morphology of VGIIc isolates, we examined selected isolates in DMEM media and after exposure to macrophages. This analysis included two VGII environmental isolates (CBS8684, CBS7750) and four of the VGIIc/novel isolates. As expected, the vast majority of the mitochondria for all six isolates were non-tubular after exposure to DMEM media alone (Figure 7B). However, after exposure to macrophages, three of the four VGIIc isolates tested showed significantly higher percentages of tubular morphology (Figure 7C). The lone VGIIc isolate that did not exhibit this morphology (EJB52) was the same isolate that also had a low IPR value, and is thus an overall outlier for the VGIIc genotype.

When the results of IPR versus percentage of cells exhibiting tubular morphology were plotted, the graph showed a statistically significant correlation of the two measures with an R^2 value of 0.85 (Figure 7D). These results further indicate that the VGIIc genotype is phenotypically similar to the Vancouver Island VGIIa/major outbreak strains. Our results also support evidence for similar mechanisms regulating the increased virulence seen in the novel VGIIc genotype. The exact roles that the mitochondrial

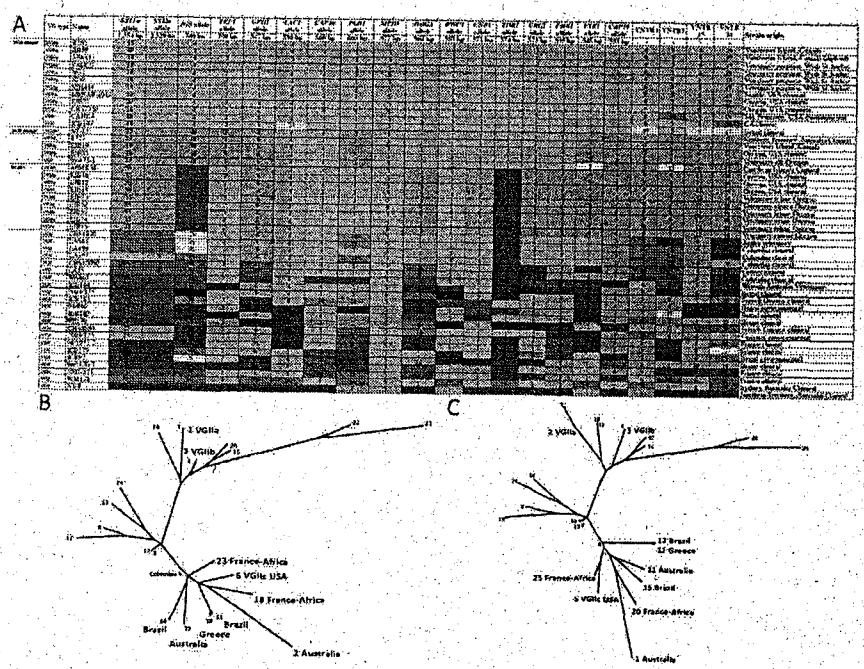

Figure 4. Expanded molecular analysis reveals increased divergence in VGIIc. A) Multilocus sequence typing analysis of 16 loci. Selected isolates from the outbreak in addition to global genotypes were selected for the expanded MLST analysis, including all nine of the VGIIc isolates available. Each unique allele is colored for each marker for visual discrimination, and each number represents a GenBank accession number (Table S2). B) A representation (ML) of the sequence data from panel A, with the exclusion of *MAT* locus linked markers (*SXI1α/SXI2a*). C) A combination of the sequence data from panel B, with the addition of the four highly variable VNTR markers.

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tubular morphology might play in virulence are not yet known. However, the distinct phenotype is clearly unique to the outbreak isolates and is correlated with an increased ability to grow and divide within host innate immune cells.

The VGIIc isolates were found to be highly virulent in the murine inhalation model of infection. Two studies were conducted to examine virulence. In the first murine experiment a total of six isolates (n = 5 animals/isolate), were examined including two VGIIc isolates (Figure 8A). The VGIIa/major isolate R265 served as a positive control for high virulence, based on prior studies [6], and the VGIIc isolates EJB15 and EJB18 showed similar virulence with this well characterized virulent isolate. Additionally, two VGIIa isolates that are not hypothesized to be from the current Vancouver Island outbreak, including NIH444, which is fully identical across 34 markers, and isolate CA1014, which differs from R265 at VNTR34, show a significant reduction in virulence compared to the high virulence isolates ($P<0.05$). Finally, in accordance with previous studies, the VGIIb/minor type strain R272 from Vancouver Island was avirulent in this model.

The analysis of virulence within the VGII genotype was extended in a second experiment, in which 12 isolates (n = 9–10 animals/isolate) were examined. This study included two VGIIa/major isolates from the outbreak zone, two VGIIb/minor isolates from the outbreak zone, five of the novel VGIIc isolates, two VGIIa-related isolates that are not part of the outbreak, and the *C. neoformans* var. *grubii* type strain, H99. The H99 isolate used (H99S) has been shown to be highly virulent in the murine model of infection [44,51].

As expected, all five of the VGIIc isolates from Oregon as well as the VGIIa/major isolates from Vancouver Island and Oregon, and the highly virulent H99 isolate exhibited a high level of virulence (median survival = 20.6 days). The VGIIb/minor isolates tested were significantly decreased in virulence compared to the more virulent VGIIa and VGIIc genotypes ($P<0.005$). The VGIIb isolate R272 was avirulent whereas the VGIIb isolate EJB53 from Oregon exhibited significantly less virulence compared to the VGIIa/major and VGIIc isolates ($P<0.005$, median survival = 46 days). Similar to the first animal study, two VGIIa isolates that differ at one or more molecular markers from the major VGIIa outbreak genotypes were also tested. The environmental isolate CBS7750 and a clinical isolate from South America ICB107 were significantly attenuated ($P<0.005$) (Figure 8B). These results

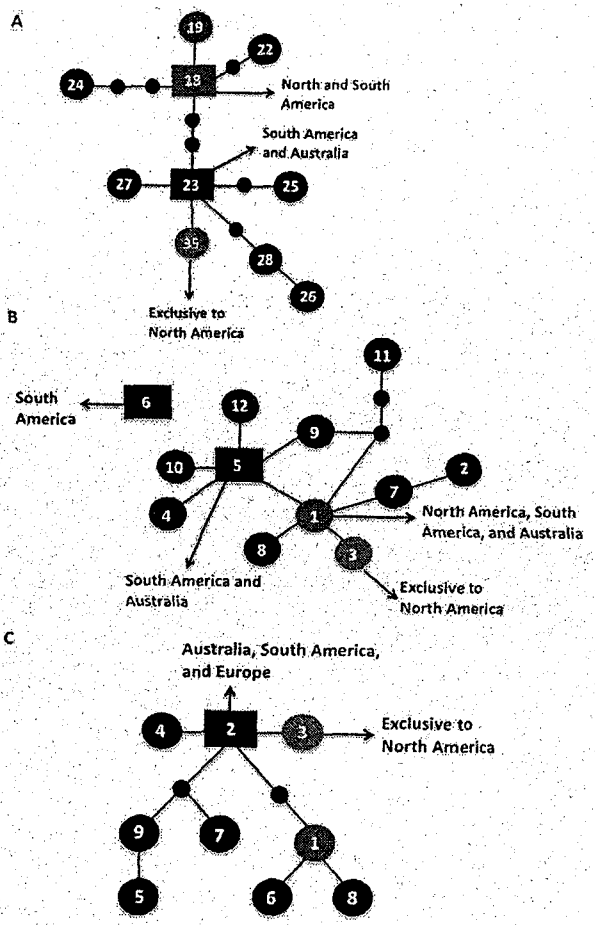

Figure 5. Haplotype networks define allele ancestry. Allele placements are indicated numerically, with the VGIIa/major genotype also represented by blue coloration, the VGIIb/minor genotype by purple coloration, and the VGIIc/novel genotype by green coloration. Large circles represent alleles extant in the population, and the small circles represent alleles that have not been recovered, or which may no longer be extant in the population. Each connecting line represents one postulated evolutionary event, with the squared allele representing the posited ancestral allele (two possible ancestral alleles depicted for *SXI1α*). A–C) Haplotype networks of the unique VGIIc alleles, *SXI1α*, *HOG1*, and *CRG1*, respectively, with geographic origins indicated.
doi:10.1371/journal.ppat.1000850.g005

provide further evidence that these are related to but distinguishable from isolates that are specific to the Vancouver Island outbreak, and subsequent United States expansion, and are decreased in ability to mount fatal infections in a mouse intranasal instillation model of infection.

The cause of infection was further evaluated by histopathological analysis of lung sections recovered from two infected animals per isolate at sacrifice. Harvested organs were processed and sectioned for slides with H&E staining. The lungs from the virulent isolates showed significant inflammation and numerous crypto-

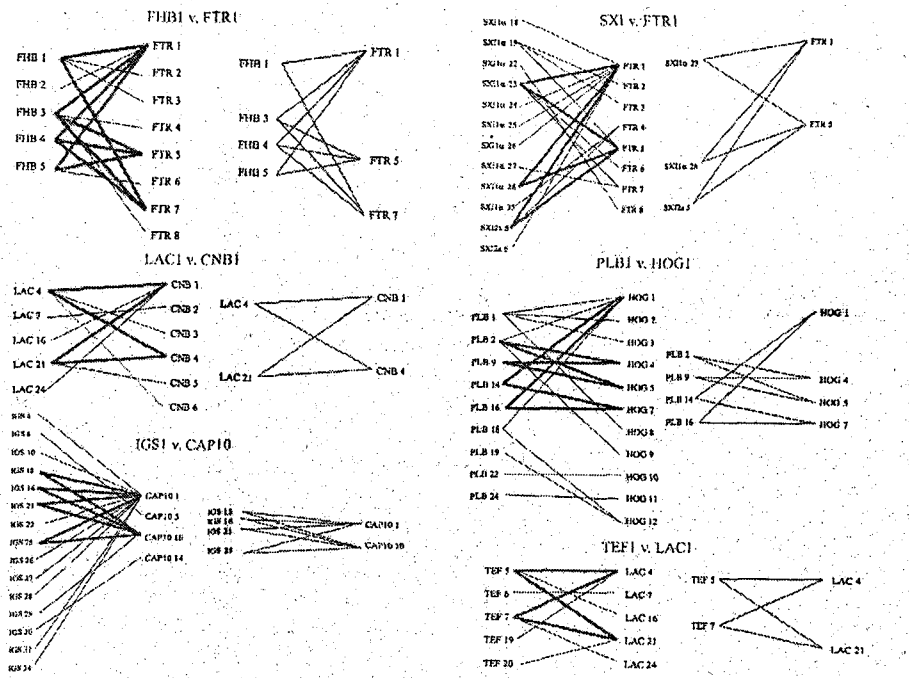

Figure 6. Evidence for recombination within the VGII molecular type. Informative paired allele graphs from VGII global isolates. An hourglass shape indicates the presence of all four possible pairs of alleles and serves as evidence for recombination. A total of 56 graphs with at least one possible recombining allele pair were generated from a set of 25 representative genotypes within the VGII molecular type, including isolates of both mating type **a** and α (see also Figure S4).
doi:10.1371/journal.ppat.1000850.g006

coccal cells dispersed throughout the alveoli, in accordance with severe pulmonary infection. Our findings show that there are no major clinical differences between pulmonary infections with the infectious genotypes VGIIa/major (Figure 8C), and the novel VGIIc genotype (Figure 8D). These results further support similar disease progression caused by these two highly virulent outbreak genotypes.

Discussion

The findings presented here document that the outbreak of *C. gattii* in Western North America is continuing to expand throughout this temperate region, and that the outbreak isolates in the United States of both the VGIIa/major genotype and the novel VGIIc genotype are clonally derived and highly virulent in host models of infection. These conclusions are based on an extensive molecular analysis of isolates collected from the United States (Table 2) and a comprehensive global collection of VGII isolates of diverse geographic origin (Figure S1), examining both conserved and divergent regions of the genome. The virulence analysis is based on assays in both murine derived macrophages and mice. These findings demonstrate that this emerging and fatal outbreak is continuing to expand, and that the virulence of these isolates is unusually high when compared to isolates of closely related but distinguishable genotypes found in other non-outbreak regions.

The continued expansion of *C. gattii* in the United States is ongoing, and the diversity of hosts increasing. Cases have been observed in urban and rural areas, and have occurred in a range of mammals [16,52]. On Vancouver Island and the mainland of British Columbia, cases have been documented in marine and terrestrial mammals including cats, dogs, porpoises, ferrets, and llamas [15,52,53]. This trend has continued in the United States, with several cases in agrarian, domestic, and wild terrestrial mammals, as well as marine mammals, adding elk, alpacas, and sheep to the aforementioned list (Table S1) [13,14,17]. The co-expansion of the outbreak among mammals and humans is significant for several reasons. Non-migratory mammals serve as sentinels for disease expansion, particularly given that isolation of *C. gattii* from the environment is difficult, and not yet successful at all in Oregon. Additionally, the threat to agricultural and domestic animals is significant and thus the need for cooperation among

Table 3. Proposed recombinant alleles and hypothesized parental contributors.

Hypothesized recombinant alleles	Isolate/genotype	Hypothesized Parental alleles	Hypothesized parental isolates/genotypes*
IGS1-4	*VGIIa*	IGS1-15	*VGIIc*, WA861, ICB184
		IGS1-16	ICB179, WM178
IGS1-30	ICB183	IGS1-22	*2004/335*
		IGS1-26	*CBS8684*, 2003/125, 98/1037-2
HOG1-2	*NT-8*	HOG1-1	*VGIIa*, VGIIb, 99/473-1, *La499*, La567, *La584*, *CBS1930*, ICB179, WM178
		HOG1-3	*VGIIc*
		HOG1-7	*96/1120-1*, 2001/571
		HOG1-4	ICB184, 2003/125, 98/1037-2
HOG1-11	ICB97	HOG1-1	*VGIIa*, VGIIb, 99/473-1, *La499*, La567, *La584*, *CBS1930*, ICB179, WM178
		HOG1-3	*VGIIc*
		HOG1-7	96/1120-1, 2001/571
		HOG1-9	97/170
CRG1-5	WA861	CRG1-1	*VGIIa*, VGIIb, 99/473-1, *La499*, La567, *La584*, *CBS1930*, 2004/335, ICB183, ICB182, *CBS8684*, 2003/125, 98/1037-2, 96/1120-1, WM178
		CRG1-6	*93/980*
		CRG1-8	ICB179
		CRG1-9	2001/571
CAP59-5	2001/571	CAP59-3	WA861, *NT-8*
		CAP59-6	ICB179
CAP59-9	97/170	CAP59-2	VGIIb, 99/473-1, *La55*, *La499*, La567, *La584*, *CBS1930*, *CBS10090*, ICB184, 2003/125, 98/1037-2
		CAP59-7	*CBS8684*
		CAP59-3	WA861, *NT-8*

* Bold indicates MATa, Italics indicates fertile representative.

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health officials is critical. Finally, the widespread spectrum of disease illustrates that the organism is likely to be pervasive in the environment, and that physicians and veterinarians should be well informed of symptoms to facilitate early diagnoses, and successful isolate collection and tracking.

A major question in the study of this outbreak is whether sexual recombination, either within or between mating types, is occurring or has occurred in the region. The possibility of meiosis is important for two reasons. The first is that sexual recombination is postulated to be a driving force for the increased virulence of the VGIIa/major genotype, supported by the discovery of a diploid VGIIa/major isolate, an intermediate in unisexual mating (all nine VGIIc/novel isolates are haploid) [6,36]. *C. gattii* has also been shown to undergo opposite sex mating in the laboratory, although this has not yet been observed to occur between two isolates of the VGII molecular type [36,54,55]. Studies in *C. neoformans* have shown that this related pathogen completes a full a-α sexual cycle in association with plants [56]. Additionally, a recent study of environmentally sampled Australian VGI isolates demonstrated evidence for recombination via both opposite and same-sex mating [50]. Taken together, available evidence indicates that both opposite and same-sex mating are naturally occurring in populations. This evidence lends support to the hypothesis that meiosis might be a factor in the forces that are driving high virulence in the outbreak region.

The second major event that results from sexual processes in the pathogenic *Cryptococcus* species is the formation of spores. Small spores ranging from 1–2 μm in diameter have been observed to be produced in large numbers as the result of opposite sex mating in both *C. neoformans* and *C. gattii* [57,58]. Studies by Lin and colleagues showed that sexual spores can be produced as the result of a meiotic process occurring between cells of the same mating type, a process referred to as unisexual or same-sex mating [59]. Several studies have shown spores to be pathogenic in animal models of infection. Two previous studies both showed evidence for virulence of *Cryptococcus* spores, and in one case provided evidence for enhanced virulence compared to yeast cells [60,61]. More recently, studies have shown that *Cryptococcus neoformans* spores are indeed virulent in the murine intranasal instillation model of infection [44,62], providing evidence that spores should be considered as infectious propagules in models examining infections, expansion, and emergence of both *C. neoformans* and *C. gattii*. Given that all of the Pacific NW isolates are α mating type, and particles small enough to be spores are present in the air [26,63], the most parsimonious model is that if these are spores, they are produced via α-α unisexual reproduction.

Our findings further indicate that mitochondria may play a significant role in the increased virulence seen in the outbreak isolates [31]. Tubular morphology and the increased ability to proliferate within immune cells indicate that the ability to proliferate and survive within host cells is fundamental to virulence. The possible role of mitochondrial involvement is intriguing and also increasingly relevant based on studies that have shown mitochondrial inheritance and recombination may impact *C. gattii* evolution, with the inheritance of the mitochondrial genome from the a mating type parent in opposite-sex mating [64,65]. Future studies in this area should address the roles that mitochondrial genes, or nuclear genes that regulate mitochondria

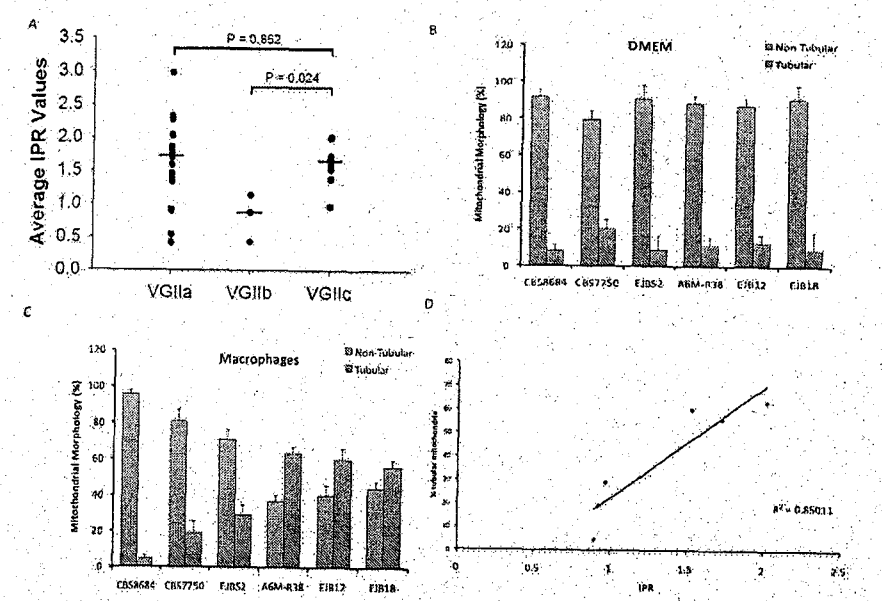

Figure 7. In vitro analyses of intracellular proliferation and mitochondrial morphology provide evidence the VGIIc genotype is hypervirulent. A) IPR rates of VGIIc isolates are similar to those from the VGIIa/major genotype and higher than those seen in the less-virulent VGIIb/minor genotype. Eight VGIIc isolates were tested individually, with the overall averages for the three primary outbreak genotypes presented. B) Percentage of cells with tubular mitochondrial morphology in DMEM. C) Percentage of cells with tubular mitochondrial morphology in macrophages. D) Linear correlation of IPR and percentage of tubular mitochondria after macrophage exposure.

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may play in the hypervirulence observed in the outbreak isolates. Furthermore, it may be that cell-cell fusion events via mating and mitochondrial exchange without meiosis or nuclear genetic exchange have played roles in recombination and virulence acquisition in naturally occurring *C. gattii* populations [64,65].

A central question in the field lies in the possible origins of the virulent genotypes. For the VGIIa and VGIIc lineages, it is clear that those are unique to the Pacific NW, and either arose there locally, or were transferred from an under-sampled region (Australia, South America, Africa). Isolates that are related to, but distinct at one or more molecular marker from VGIIa have been identified in San Francisco (CBS7750), southern California (CA1014), and South America (ICB107). However, in each of these cases, the isolates are not identical with the VGIIa/major isolates from the Pacific NW. Whether the outbreak isolates are derived from these isolates, or alternatively that these isolates are derived from the outbreak lineage is at present unclear. In the VGIIb/minor outbreak lineage, isolates from Australia are identical at all 30 MLST loci and four VNTRs analyzed, and the most parsimonious model is that the two are directly related. While it is conceivable that both the Australian and the Vancouver Island VGIIb/minor genotype isolates were dispersed independently from another geographic locale, until isolates are identified conclusively from another locale the most parsimonious model is transfer from Australia to the Pacific NW. We note that a single isolate with a related but distinct genotype (isolate 99/473) from the Caribbean has been identified; and other isolates have been reported to share the VGIIb genotype but have been analyzed at a limited number of MLST markers (n = 7) which is insufficient to establish how closely related these isolates are to the outbreak VGIIb/minor genotype strains [29]. The origins of VGIIc are unclear, with the genotype possibly arriving in the Pacific NW from South America, Africa, Europe, or Australia. Alternatively, this novel unique genotype may have arisen locally.

As for the geographic origins of VGII diversity, this also remains to be established and may involve populations in Australia, South America, and Africa. It is clear that there is considerable diversity among isolates from South America. As we originally proposed as an alternative model [6], and has been independently presented by other investigators (W. Meyer, T. Boekhout, JP Xu, pers. comm.), South America may represent a source of diversity and ongoing generation of novel isolates. Analysis of 8 MLST loci in this study indicates that in South America and the Caribbean there are 14 genotypes seen in 21 isolates, while in North America only 3 genotypes have been observed through the analysis of 64 isolates (Figure S1). Additionally, there is accumulating evidence that fertile isolates of both a and α mating type are present in South America [29], and thus ongoing a-α opposite sex mating may be occurring there. It is also clear that a unique set of VGII isolates are circulating in Australia, and there is evidence for ongoing

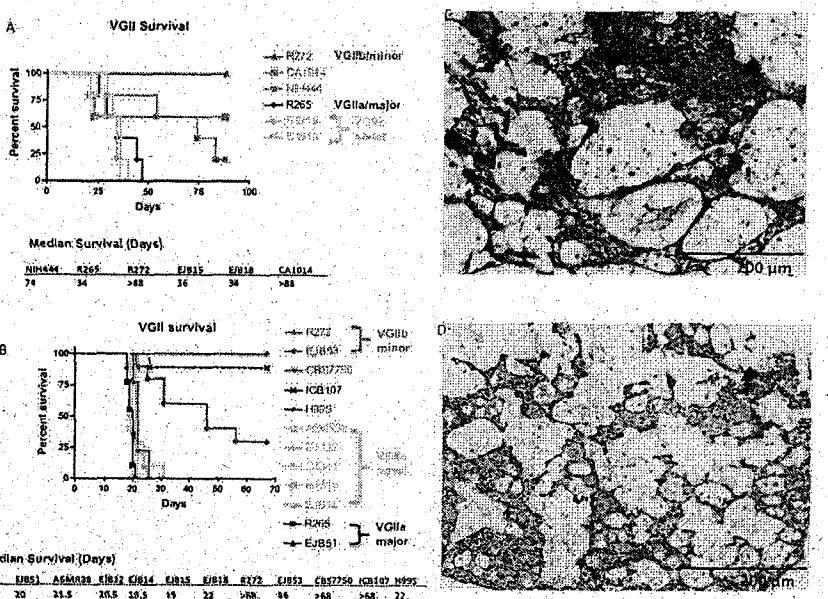

Figure 8. Isolates from the United States outbreak are hypervirulent. A) Groups of five animals were each infected with an infectious inoculum of 1.0×10^5 cells of VGIIa isolates R265, CA1014, or NIH444, VGIIb isolate R272, or VGIIc isolates EJB15 or EJB18. B) Groups of nine or ten animals were each infected with an infectious inoculum of 5.0×10^4 cells of VGIIa isolates R265, EJB51, CBS7750, or ICB107, VGIIb isolates R272 or EJB53, VGIIc isolates A6MR38, EJB12, EJB14, EJB15, or EJB18, or *C. neoformans* var. *grubii* isolate H99. C–D) Representative H&E stained histopathology slides from lung sections of severely morbid sacrificed animals from the VGIIa/major (R265) (C) and VGIIc (EJB18) (D) genotypes (sections from animals in panel B of this Figure).
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recombination in α only and a-α populations, suggesting that mating contributes to the generation of diversity in Australia [36,49,54,55,66,67]. Finally, the analysis of global VGII isolates reveals genetic diversity in Africa, and given the recent findings that *C. neoformans* likely originated in sub-Saharan Africa (A. Litvintseva and T. Mitchell, pers. Comm.), further analysis of African *C. gattii* isolates is clearly warranted.

It remains possible that South America, Africa, or both represent the ancestral populations of *C. gattii*, and that more recent dispersal events from other established populations (for example, from Australia to the Pacific Northwest) have occurred to contribute to the outbreak. As yet, all of the isolates found in the Pacific Northwest are α mating type. Thus, if sexual reproduction is occurring in the Pacific Northwest, it would appear to involve same-sex mating occurring under environmental conditions. Recent studies have documented that *C. neoformans* and *C. gattii* are stimulated to undergo opposite-sex mating in laboratory conditions that simulate environmental niches (pigeon guano medium, co-culture with plants) and thus similar conditions may be necessary in nature [56,68]. Overall, both the VGIIa/major and the VGIIc/novel genotypes contain a number of MLST loci that are thus far restricted to these lineages, and their origins remain to be identified.

Independently of the variables leading up to and influencing this outbreak, the major concern is and continues to be the inexorable expansion throughout the region. From 1999 through 2003, the cases were largely restricted to Vancouver Island. Between 2003 and 2006, the outbreak expanded into neighboring mainland British Columbia and then into Washington and Oregon from 2005 to 2009. Based on this historical trajectory of expansion, the outbreak may continue to expand into the neighboring region of Northern California, and possibly further.

The rising incidence of cryptococcosis cases in humans and animals highlights the need for enhanced awareness in the region, and those regions that may potentially become involved. While rare, little is currently known about how or why specific humans and animals become infected. Increased vigilance may decrease the time from infection to diagnosis, and thus lead to more effective treatment and a reduction in mortality rates. The potential dangers of travel-associated risks should be noted, as a growing number of cases attributable to travel within the Pacific NW region have been documented [69,70]. Northern California has similar temperate climates to endemic regions within Oregon, leading to the hypothesis that the emergence may expand there, while expansion eastward may be limited by winters with average temperatures often below freezing [17].

The expansion of the outbreak into California is plausible based on several studies documenting the presence of *C. gattii* throughout the state and in Mexico. *C. gattii* molecular type VGII was environmentally isolated in the San Francisco area in 1990 (isolate CBS7750) [48], and there have also been two confirmed and one travel-associated case of *C. gattii* molecular type VGI in California. Of the VGI cases, one occurred in a male Atlantic bottlenose dolphin in San Diego, one was isolated from a liver transplant recipient in San Francisco, and the other from an otherwise healthy patient in North Carolina with travel history to the San Francisco region [71,72,73]. In addition *C. gattii* has been reported in southern California among a cohort of HIV/AIDS patients [74]. Recently, studies of clinical isolates from Mexico revealed all four molecular types of *C. gattii* to be present [75]. Taken together, the hypothesis that the virulent isolates from the Pacific NW will expand into California must be considered by both physicians and public health officials.

During the coming years, monitoring and researching the outbreak expansion as a multidisciplinary effort will be critical. The ability to bring diverse groups of professionals interested in *C. gattii* expansion has been greatly facilitated through the formation of the *Cryptococcus gattii* working group of the Pacific Northwest [17]. From a research standpoint, further examination of the molecular mechanisms underlying the increased virulence in both VGIIa/major and VGIIc/novel will be useful for the development of aggressive treatments that may be needed. Furthermore, increased efforts to determine the ecology and population dynamics of *C. gattii* in the region, and elucidating the evolutionary history of the VGIIc genotype will be critical to gain further insights into the origins of this unprecedented and frequently fatal fungal outbreak.

Supporting Information

Figure S1 MLST of all VGII isolates used in the study and the four out-group isolates used in the phylogenetic analysis.
Found at: doi:10.1371/journal.ppat.1000850.s001 (0.07 MB PDF)

Figure S2 MLST analysis of Vancouver Island isolates at 2 loci. These were chosen to determine if any of the isolates might have belonged to the VGIIc group.
Found at: doi:10.1371/journal.ppat.1000850.s002 (0.02 MB PDF)

Figure S3 TCS haplotype networks for the thirteen alleles not represented in Figure 5 of the main text.
Found at: doi:10.1371/journal.ppat.1000850.s003 (0.15 MB PDF)

Figure S4 All paired allele graphs from VGII global isolates generated during the analysis. Isolates of both mating type a and α were included. In addition, a group of ten isolates, all α, from South America and Africa appeared most commonly as recombinant partners and are illustrated.
Found at: doi:10.1371/journal.ppat.1000850.s004 (0.12 MB PDF)

Figure S5 Allelic recombination analysis for 15 loci indicates that 11 are likely derived from consecutive and/or independent mutations within the population. The four other loci show at least one hybrid allele that may be the result of a recombination event between two proposed parental alleles in the global VGII population. Squared alleles represent likely recombinants, while circled alleles indicate proposed parental contributors. Each of the possible contributors is indicated by a respective color.
Found at: doi:10.1371/journal.ppat.1000850.s005 (1.34 MB PDF)

Table S1 Primers used in the study.
Found at: doi:10.1371/journal.ppat.1000850.s006 (0.03 MB XLS)

Table S2 GenBank accession numbers for all of the MLST and VNTR alleles represented in the text and figures.
Found at: doi:10.1371/journal.ppat.1000850.s007 (0.05 MB XLS)

Table S3 Detailed sequence type information from Figure 3.
Found at: doi:10.1371/journal.ppat.1000850.s008 (0.03 MB DOC)

Table S4 Detailed sequence type information from Figure 4B and Figure 4C.
Found at: doi:10.1371/journal.ppat.1000850.s009 (0.02 MB XLS)

Table S5 Mating properties of selected VGII isolates.
Found at: doi:10.1371/journal.ppat.1000850.s010 (0.02 MB XLS)

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Author Contributions

Conceived and designed the experiments: EJB WL YL DAC VC RCM JH. Performed the experiments: EJB WL YL HM KV PR. Analyzed the data: EJB WL YL HM KV PR DAC RJB RCM JH. Contributed reagents/materials/analysis tools: VC RJB JH. Wrote the paper: EJB WL YL JH.

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研究報告の概要	目的：「プロテアーゼ感受性プリオン症(PrPr)」の 2 つの新しい遺伝子型を報告する。これは 2008 年に記載された新型のプリオン病で、被験者 11 人はプリオン蛋白質(PrP)遺伝子のコドン 129 が全員バリン同型接合(129VV)だった。2 つの新しい PSPr は、メチオニン同型接合(129MM)とメチオニン/バリン異型接合(129MV)の個人である。 方法：129MM、129MV、129VV の被験者 15 人は国立プリオン病病理監視センターで臨床、組織病理学的、免疫組織化学、遺伝子型、PrP 特性で比較評価を受けた。 結果：潜病期間(22 から 45 ヶ月の間)は、129VV と 129MV の被験者で有意に異なった。PrP 電気泳動プロフィルと共に他の特徴の表現型の機能は同様だったが、3 つの 129 遺伝子型で区別できる。主な違いは、疾患関連 PrP のプロテアーゼ消化の感受性にあり、129VV では高度であるが、129MV と 129MM では遙かに低いか、全くない。この違いは本来の命名を変え、「可変プロテアーゼ感受性プリオン症(VPSPr)」になった。被験者は誰も PrP 遺伝子コドン領域の変異はなかった。 解釈：3 つの 129 遺伝子型が全員関係し、区別でき、表現型として関係するので、VPSPr は二番目の孤発性プリオン蛋白質疾患になる。この特徴は 1920 年に報告したクロイツフェルト・ヤコブ病によく似ていた。しかし、異常プリオン蛋白質の特性は VPSPr は典型的なプリオン病とは異なることを示唆している。おそらく、ゲルストマン・ストロイスラー・シャインカー疾患の亜型と類似している。					使用上の注意記載状況・その他参考事項等 代表としてノイアート静注用 500 単位の記載を示す。 2. 重要な基本的注意 (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。	
	報告企業の意見			今後の対応					
「可変プロテアーゼ感受性プリオン症(VPSPr)」という、新規の孤発性プリオン病についての報告である。 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、弊社の原料血漿採取国である日本及び米国では、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、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。					

22

ORIGINAL ARTICLE

Variably Protease-Sensitive Prionopathy: A New Sporadic Disease of the Prion Protein

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Objective: The objective of the study is to report 2 new genotypic forms of protease-sensitive prionopathy (PSPr), a novel prion disease described in 2008, in 11 subjects all homozygous for valine at codon 129 of the prion protein (PrP) gene (129VV). The 2 new PSPr forms affect individuals who are either homozygous for methionine (129MM) or heterozygous for methionine/valine (129MV).
Methods: Fifteen affected subjects with 129MM, 129MV, and 129VV underwent comparative evaluation at the National Prion Disease Pathology Surveillance Center for clinical, histopathologic, immunohistochemical, genotypical, and PrP characteristics.
Results: Disease duration (between 22 and 45 months) was significantly different in the 129VV and 129MV subjects. Most other phenotypic features along with the PrP electrophoretic profile were similar but distinguishable in the 3 129 genotypes. A major difference laid in the sensitivity to protease digestion of the disease-associated PrP, which was high in 129VV but much lower, or altogether lacking, in 129MV and 129MM. This difference prompted the substitution of the original designation with "variably protease-sensitive prionopathy" (VPSPr). None of the subjects had mutations in the PrP gene coding region.
Interpretation: Because all 3 129 genotypes are involved, and are associated with distinguishable phenotypes, VPSPr becomes the second sporadic prion protein disease with this feature after Creutzfeldt-Jakob disease, originally reported in 1920. However, the characteristics of the abnormal prion protein suggest that VPSPr is different from typical prion diseases, and perhaps more akin to subtypes of Gerstmann-Sträussler-Scheinker disease.

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Human prion diseases are prominently heterogeneous. In sporadic Creutzfeldt-Jakob disease (sCJD), the most prevalent prion disease, heterogeneity is largely predicated on the common methionine (M)/valine (V) polymorphism at codon 129 of the prion protein (PrP) gene and the disease-associated PrP (PrP^{Dis}) that are distinguished in types 1 and 2 based on the electrophoretic mobility of their protease-resistant regions.[1]

However, despite this remarkable heterogeneity, all well-established sporadic prion diseases (here operationally defined as nonacquired prion diseases free of mutations in the PrP gene coding region) have been shown to share the same basic pathogenetic mechanism; PrP^{Dis} interacts with the normal or cellular PrP and converts it into PrP^{Dis}, triggering an autocatalytic process that leads to the accumulation of PrP^{Dis} and ultimately to the clinical disease.[2]

In 2008, we described 11 cases affected by a new disease involving PrP; we named this disease protease-sensitive prionopathy (PSPr).[3] Subsequently, 2 additional cases of PSPr have been independently reported.[4,5] PSPr differed from known sporadic prion diseases in the clinical presentation, in the histopathologic and immunohistochemical features, and in the basic characteristics of the PrP^{Dis}. Furthermore, all 11 cases had the 129VV genotype and no mutation in the PrP gene open reading frame (ORF).

We now report 15 additional cases, all of which bear features of the PSPr as originally reported. However, the new cases also include, in addition to new 129VV subjects, individuals who are 129MV heterozygous and 129MM homozygous. Although the affected subjects belonging to the 3 genotypes share several important characteristics, they also display basic variations that allow the 3 corresponding phenotypes to be distinguished. Therefore, the new cases show that the disease originally described as PSPr, like sCJD, affects all 3 129 genotypes and to some extent mimics the 129-related phenotypic heterogeneity of sCJD, although the PSPr characteristics underline basic differences from sCJD and similarities with Gerstmann-Sträussler-Scheinker disease (GSS), a rare phenotype, which to date has been reported as exclusively associated with PrP gene mutations. In view of the increased protease-resistance of the PrP^{Dis} associated with the new 129 genotypes compared to that of the 129VV cases, we propose to revise the original PSPr label to VPSPr or "variably protease-sensitive prionopathy." Parts of these findings have been presented previously.[6–9]

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Subjects and Methods

Subjects

A total of 15 affected subjects, including 3 129MM, 6 129MV, and 6 previously unreported 129VV, were examined. Thirteen affected subjects were referred to the National Prion Disease Pathology Surveillance Center (NPDPSC) (Cleveland, OH) between 2002 and 2010. All cases were symptomatic except 1 of the 129MM subjects, who died suddenly of heart problems while participating in a dementia study as a negative control, underwent autopsy, and was referred to the NPDPSC because it was noted to have spongiform degeneration (SD) on histological examination. One 129MM subject was received by Dr Fabrizio Tagliavini[10] (National Neurological Institute, Instituto Nazionale Neurologico Carlo Besta, Milan, Italy), and 1 129MV subject was received by Dr Piero Parchi (Department of Neurological Sciences, University of Bologna, Dipartimento di Scienze Neurologiche, Università di Bologna, Bologna, Italy). All the subjects including those serving as positive control as indicated were examined at autopsy following analyses of fixed and frozen tissues. Consent was obtained for using tissues for research, including genetic analyses.

Tissue Processing

Fixed and frozen brain tissues were processed as previously described; a different procedure was followed for the case received from Dr Tagliavini.[3,10–12]

Histopathology and Immunohistochemistry

Samples obtained from up to 18 brain regions were processed according to previously described procedures.[3,12] Lesion profiles were constructed using semiquantitative evaluation of SD and astrogliosis in 11 brain regions from 10 subjects, including the 3 129 genotypes. SD and astrogliosis were scored (Fig 1), and the scores from each of the brain regions were summed for each subject separately; values were averaged, their standard deviations determined, and they were plotted according to the brain region.[3,12] Vacuoles with >4μm diameter were measured individually on random photomicrographs of frontal neocortex (10/subject, ×180) using Spotsoftware version 4.6 after calibration (Diagnostic Instruments, Sterling Heights, MI).[3]

Sections from the frontal and occipital neocortices, hippocampus, basal ganglia, thalamus, cerebellar hemisphere, and midbrain were processed for PrP immunohistochemistry with the monoclonal antibody (mAb) 3F4 or 1E4 (Cell Sciences, Canton, MA).[11–17] Selected brain regions were also immunostained with the mAbs 4G8 to amyloid β or PHF1 to the tau protein.[3]

Molecular Genetics

The entire PrP ORF was amplified by polymerase chain reaction using genomic DNA (extracted from unfixed brain tissue or blood) and the primers 42F (CATAACTTAGGGTCACATTTGTCC) and 45R (CCAGATTAACCAATGGTTATTTGC); sequencing was done directly or after cloning into plasmid pSTBlue 1 (Novagen, Madison, WI) by automated sequencing.[13]

Prion Protein Characterization

REAGENTS AND ANTIBODIES. Phenylmethylsulfonyl fluoride was purchased from Sigma Chemical Co. (St. Louis, MO). Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA) and used following the

manufacturers protocol. Reagents for enhanced chemiluminescence (ECL Plus) were from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies to various sequences of human PrP included anti-C, a rabbit antiserum (220–231), 3F4, a mouse mAb (106–110), and 1E4, a mAb (97–108).[3,14–17]

BRAIN HOMOGENIZATION. The 10% (weight/volume) brain homogenates were prepared in 9 volumes of lysis buffer (100mM Tris, 150 mM NaCl, 0.5% Nonidet P40, 0.5% deoxycholate, 5mM ethylenediaminetetraacetic acid, pH 8.0) on ice using pestles with Eppendorf tubes driven by a cordless motor as previously described.[14] When required, brain homogenates were centrifuged at 1,000 × *g* for 10 minutes at 4°C to collect supernatant.

IMMUNOBLOT ANALYSIS. Samples were resolved on 15% Tris-HCl Criterion precast gels (Bio-Rad Laboratories, Hercules, CA) for gel electrophoresis and Western blotting as described previously.[15] The proteins on the gels were transferred to Immobilon-P membrane polyvinylidene fluoride (Millipore, Billerica, MA) for 2 hours at 70V. For probing PrP, the membranes were incubated for 2 hours at room temperature with anti-PrP antibodies. Following incubation with horseradish peroxidase-conjugated sheep antimouse immunoglobulin G (IgG) or donkey antirabbit IgG at 1:3,000, the PrP bands were visualized on Kodak film (Eastman Kodak, Rochester, NY) by ECL Plus as described by the manufacturer.

Results

Clinical Features

The cases, grouped according to the 129 genotype, demographics, and clinical data and tests, along with the previous 129VV cases, are summarized in the Table.

In 129VV cases, the presentation was characterized by 1 or more components of a triad comprising psychiatric signs, in the form of behavior and mood changes, speech deficit, and cognitive impairment. Behavior and mood changes, expressed as disinhibition, euphoria, and impulsivity or loss of interest and apathy, were the most frequent (80% of the cases). Language deficits, observed in half of the cases, were characterized by anomic or semantic aphasia, or by dysarthria. Cognitive impairment, mostly of the frontal lobe type, was present at onset in 50% of the cases, alone or together with the behavioral changes and language deficits.

In the 129MV subtype, psychiatric signs were often associated with parkinsonism, followed by ataxia and myoclonus, whereas aphasia was rare; in these cases, the mean age at onset (72 years) and duration (45 months) of the disease were the most advanced and the longest, respectively, of the 3 subtypes and the duration was significantly different from those of the 129VV genotype ($p < 0.017$).

Both symptomatic 129MM subjects (1 died apparently before clinical onset of disease) presented with Parkinsonism and ataxia followed by progressive diffuse cognitive impairment and myoclonus; aphasia was reported in 1 case, but neither showed psychiatric symptoms.

As for the diagnostic tests, just 1 VV case showed signal changes consistent with CJD on magnetic resonance imaging and electroencephalography; all the other cases revealed various degrees of brain atrophy and diffuse slowing of cerebral electrical activity.

Familial occurrence of dementia was reported in about 50% (7/14 available family histories) of the 129VV cases (1 case in the present series), and in 1 129MM, but not in the 129MV genotype.

Histopathology

The hallmark common to all 129 genotypes was the presence of moderate SD comprising vacuoles in the major cerebral regions, which were relatively larger than those observed in sCJDMM1 but overall smaller than those of sCJDMM2 (see Fig 1A–E and Fig 1A-C of Gamberti et al[3]). Occasionally, the molecular layer of the cerebellum contained small homogeneous formations, with the appearance of microplaques in the 129VV and 129MV cases (see Fig 1F, G). On average, all these lesions were more severe in the 129VV and 129MV than in the 129MM cases (see Fig 1E).

The pattern of PrP immunostaining was slightly different in the 3 genotypes.

In the 129VV cases, the PrP immunostaining, as originally described, was targetlike in the cerebrum (Fig 2A) and dotlike in the cerebellar molecular layer (see Fig 2B).

In the 129MV genotype, the targetlike pattern was less recognizable (see Fig 2C). The cerebellar molecular layer showed a more plaquelike immunostaining pattern (see Fig 2D).

In the 129MM subjects, the predominant immunostaining pattern was plaquelike (see Fig 2E). The cerebellum occasionally showed small plaquelike formations (see Fig 2F).

With mAb 1E4, the patterns of PrP immunostaining were similar to those revealed by 3F4 (data not shown).

Various degrees of amyloid β immunostaining, apparently age-correlated, were also observed (data not shown).

Characterization of PrP^{Dis} in the 3 129 Genotypes

PRP^{Dis} ELECTROPHORETIC PROFILE AND PROTEINASE K RESISTANCE. The ladderlike electrophoretic profile of the proteinase K (PK)-resistant PrP^{Dis} fragments, the

TABLE: Summary of Cases

Codon 129	Distribution, % (No.)	Onset, $\bar{x} \pm$ SD (range)	Duration, mo ± SD (range)	Main Neurological Signs: Psychiatric,[a] %	Aphasia, %	Parkinsonism, %	Ataxia, %	Myoclonus,[b] %	Frontal Type Dementia	Diagnostic Tests: 14.3.3, Positive/ Total or %	EEG, Typical/ Total or %	MRI,[c] Typical/ Total or %	Family History of Dementia
VPSPr													
MM	12 (3)[d]	64 & 78[e]	41 & 50	0	50	100	50	100	0	1/2	1/2	0/2	1/2
MV	23 (6)	72 ± 7[f] (65–81)	45 ± 24[g] (7–72)	83	17	67	67	40	50	0/2	0/2	0/6	0/5
VV	65 (17)[h]	65 ± 8[e,f] (48–77)	25 ± 17[g] (10–60)	71	47	30	71	5	60	1/7	0/14	1/17	7/13
Total % distribution	100	67 ± 10 (48–81)	30 ± 21 (7–72)	68	48	48	64	20	52	2/12	1/18	1/25	8/20
sCJD[i]													
MM1	68	65 (42–91)	4(1–18)	28	23	7	33	97	NA	95%	80%	75%	—
MV1	2	62 (51–72)	12		25	0	75	100	NA		71%		
VV1	1	37 (19–55)	21(10–49)	0	33	33	0	67	NA	100%	0%	100%	—
MM2	2	64 (49–77)	16 (9–36)	0	33		17	67	NA	75%	0%	43%	—
MV2	9	60 (41–81)	17 (5–72)	34	11	22	81	77	NA	80%	7%	86%	—
VV2	16		6 (3–18)	19	0	6	100	66	NA		7%	70%	—
Totals	93[j]	63	6	26	18	8	49	87	NA	89%	56%	72%	

[a] Psychiatric symptoms include depression, psychosis, and personality/behavioral changes.
[b] Generally appreciable in the ataxic cases.
[c] Presence of 2 MRI patterns showed significant cerebral atrophy.
[d] One of the 3 129MM subjects who died of accidental causes before onset of clinical disease has been excluded.
[e] $p < 0.025$.
[f] $p < 0.05$ (statistical analysis by GraphPad [La Jolla, CA] Prism 3 software).
[g] Including the 11 129VV cases previously published.[3]
[h] Data adapted from Gamberti et al,[3] Parchi et al,[17] and Zou et al,[17] cases with co-occurrence of disease-associated prion protein type 1 and 2 have been omitted.[14,18,19]
[i] Does not include sporadic fatal insomnia (2%).[20]
[j] 5 cases.
SD = standard deviation; EEG = electroencephalogram; MRI = magnetic resonance imaging; VPSPr = variably protease-sensitive prionopathy; M = methionine; V = valine; sCJD = sporadic Creutzfeldt-Jakob disease.

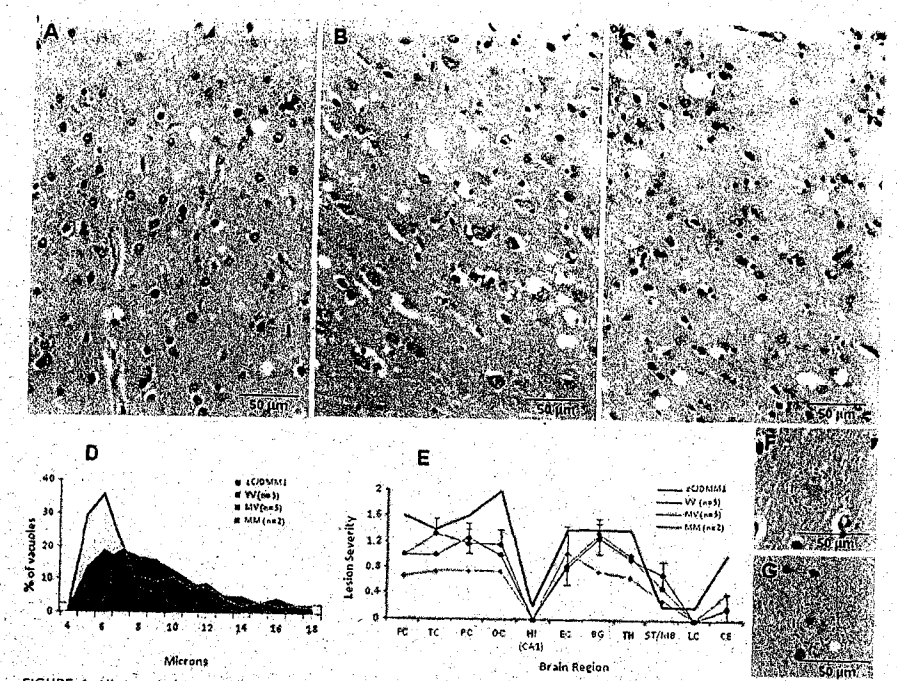

FIGURE 1: Histopathology with vacuole size and lesion profiles in the cases belonging to the 3 129 genotypes of variably protease-sensitive prionopathy (VPSPr). The spongiform degeneration is qualitatively similar in all 3 129 genotypes (A, 129VV; B, 129MV; C, 129MM). (D) As originally shown in the 129VV cases,[3] the spongiform degeneration is made of a significant percentage of relatively large and midsize vacuoles on average significantly larger than those of common sporadic Creutzfeldt-Jakob disease (sCJD) subtypes (diameters: VPSPr [combined] 9.3 ± 3.4μ vs sCJDMM1 5.8 ±1.2μ; $p < 0.0001$ [Student t test]), resulting in an elongated vacuole size distribution in the vacuole size histogram. (E) The lesion profiles are very similar in the 3 129 genotypes, but show less severe lesions in the 129MM genotype than in the 129VV and 129MV genotypes. FC, TC, PC, and QC= frontal, temporal, parietal, and occipital cortices; HI = CA1 of hippocampus; EC = entorhinal cortex; BG = basal ganglia; TH = thalamus (medial-dorsal nucleus); ST/MB = striatum/midbrain; LC = pons (locus coeruleus); CE = cerebellar cortex. The vertical bars refer to standard deviations. Spongiform degeneration was scored on a 0 to 4 scale (0, not detectable; 1, mild; 2, moderate; 3, severe; and 4, confluent), and astrogliosis on a 0 to 3 scale (0, not detectable; 1, mild; 2, moderate, and 3, severe). (F, G) Homogeneous micro deposits with the appearance of plaques were observed in the molecular layer of the cerebellum in some cases associated with the 129VV (F) and 129MV (G) genotypes, but not in the 3 129MM cases. (A–C, F, G) Hematoxylin & eosin. M = methionine; V = valine.

distinctive feature of the PSPr 129VV cases, was shared by all the affected subjects belonging to the 129MM and 129MV genotypes, although, due to the higher PK resistance, the representation of most PrP^{Dis} fragments was greater in 129MM and 129MV than in 129VV (Fig 3A).[3] The ladderlike profile demonstrated with 1E4 consisted of 5 major bands migrating at approximately 26kDa, 23kDa, 20kDa, 17kDa, and 7kDa (hereafter identified as VPSPr26, VPSPr23, VPSPr20, VPSPr17, and VPSPr7) (see Fig 3A). In contrast, PrP^{Dis} types 1 and 2 from sCJD formed the classical pattern of 3 bands migrating at 32/30kDa, 28/26kDa, and 21/19kDa (see Fig 3A).

PrP^{Dis} preparations from the 3 genotypes were probed with mAb 1E4 or 3F4 after treatment with various amounts of PK. When PrP^{Dis} fragments were considered all together, both 1E4 and 3F4 confirmed the relatively high PK resistance of PrP^{Dis} in the 129MM cases, intermediate in the 129MV cases, and low or entirely lacking in the 129VV cases (see Fig 3). However, both mAbs also showed the heterogeneous resistance of the individual fragments to PK, which was confirmed with

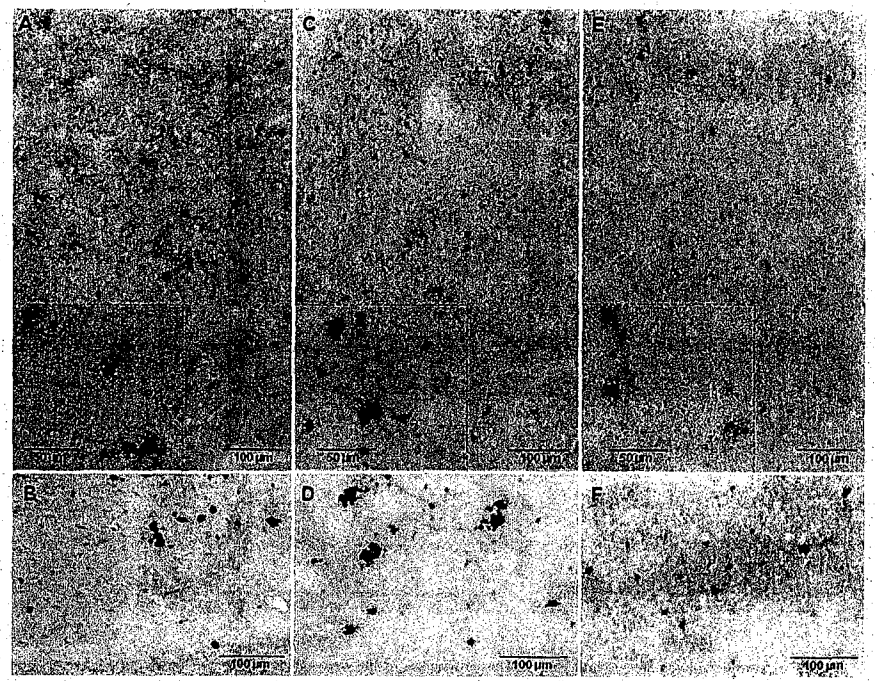

FIGURE 2: Prion protein immunohistochemistry in the 3 variably protease-sensitive prionopathy 129 genotypes. The cerebral cortex (A, C, E) and cerebellar molecular layer (B, D, F) best exemplify the predominant immunostaining patterns. (A) The pattern in the 129VV genotype is often targetlike, with a larger stained granule or clusters of granules surrounded by smaller granules in a focal or more diffuse background of punctate or synaptic staining (*inset*: higher magnification of the same cortical region). (B) The molecular layer of the cerebellum shows relatively large granules that are often compact and intensely stained. (C) In the 129MV genotype, the targetlike pattern is generally less obvious, as large granules are more often isolated; focal or larger areas of synaptic staining are also present (*inset*: as above). (D) In the cerebellum, the granules are fewer, are more loose, and have a plaquelike appearance. (E) The 129MM genotype often shows a plaquelike immunostaining pattern (*inset*: as above). (F) The cerebellum shows small formations. Immunostaining was done with monoclonal antibody 3F4. M = methionine; V = valine.

PK titration experiments. These analyses showed that the VPSPr7 fragment was highly PK-resistant in all 3 genotypes. In contrast, the other 4 fragments appeared to follow 2 distinct patterns, which were similar and involved pairs of the same fragments in both 129MV and 129MM; VPSPr26 and VPSPr20 increased and decreased rapidly in amount peaking at 10μg/ml of PK and generated fairly narrow bell-shaped curves. In contrast, both PSPr23 and PSPr17 increased at a lower rate, peaked between 25 and 50μg/ml of PK, and remained relatively well represented even at 100μg/ml of PK. The representations of the 2 pairs of fragments were significantly different at 100μ/ml PK concentration in both 129 genotypes (129MM, $p < 0.02$; 129MV, $p < 0.005$). As expected, the PK resistance of the 129VV fragments was much lower, except for VPSPr7. Combined, the immunoblots and quantitative analyses argue that VPSPr23 and VPSPr17 have the strongest resistance to PK and likely form secondarily from VPSPr26 and VPSPr20 following treatment with high PK concentrations. It has to be noted, however, that the PK sensitivity of the 129VV preparations was in part related to the mAb used. When probing with 1E4 instead of 3F4, all fragments present in the VPSPr-129MM and -129MV preparations were also detectable in the preparations from the 129VV genotype, even if they displayed different ratios. Therefore,

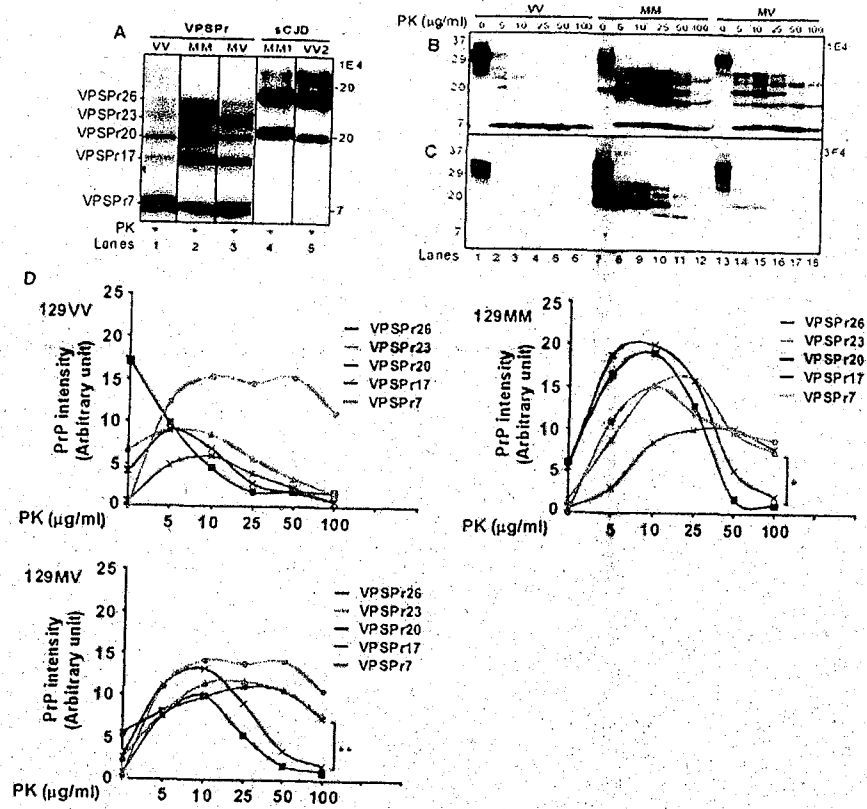

FIGURE 3: Electrophoretic profiles and proteinase K (PK) titration of PK-resistant disease-associated prion protein (PrP^{Dis}) from variably protease-sensitive prionopathy (VPSPr) associated with the 129VV, 129MM, or 129MV genotype. (A) The Western blots of the total brain homogenates (BHs) treated with 25μg/ml of PK and probed with the monoclonal antibody 1E4 reveal 5 PrP bands migrating approximately to 26kDa, 23kDa, 20kDa, 17kDa, and 7kDa, forming a ladderlike pattern in all 3 (129VV, 129MM, and 129MV) genotypes of VPSPr (VPSPr26, VPSPr23, VPSPr20, VPSPr17, and VPSPr7) *(lanes 1–3)*. The faint band that migrates at approximately 30kDa in VPSPr-129VV *(lane 1)* likely represents the incomplete PK digestion of the normal diglycosylated, N-terminus truncated PrP fragment or associated monoglycosylated full-length PrP. In contrast, BHs from sporadic Creutzfeldt-Jakob disease (sCJD) associated with the 129MM genotype and the PrP^{Dis} type 1 (sCJDMM1) or sCJDVV2 (sCJD with the 129VV genotype and PrP^{Dis} type 2) show the typical 3 PK-resistant PrP fragments of type 1 and 2 migrating between 31kDa and 19kDa *(lanes 4 and 5)*. (B, C) PK titration of PrP^{Dis}. Brain homogenates from 129VV, 129MM, and 129MV genotypes were treated with PK at various concentrations between 0 and 100μg/ml. (B) Probed with 1E4. (C) Probed with 3F4. (D) PK titration with quantitative analysis of the individual VPSPr fragments. The curves represent the relative amounts of the individual VPSPr fragments at increasing PK concentrations (0–100μg/ml) after probing the immunoblots with 1E4 in each of the 3 129 genotypes. The relative representations of the bands corresponding to the VPSPr fragments were determined by densitometry and expressed as averages of 129VV (n = 3), 129MM (n = 2), and 129MV cases (n = 3). Comparative analysis of the curves from each of the 3 129 genotypes confirms the PK sensitivity of all the fragments in 129VV cases, with the exception of VPSPr7, which is resistant to PK in all 3 genotypes. The remaining 4 fragments follow similar patterns in both the 129MM and 129MV genotypes; VPSPr26 and VPSPr20 form rapidly but are digested at PK concentrations >10μg/ml; VPSPr23 and VPSPr17 are resistant up to 100μg/ml of PK (*$p < 0.02$; **$p < 0.005$). M = methionine; V = valine.

the PK treatment might not only break down the PrP^{Dis} associated with the 129VV genotypic form, but also generate fragments relatively undetectable by the mAb 3F4. Alternatively, PrP^{Dis} associated with VPSPr-129VV might have a low immunoreactivity with 3F4, even without PK treatment.

IDENTIFICATION OF THE PRP^{DIS} CORE FRAGMENTS AND THEIR COMPARISON WITH THOSE OF THE GSS VARIANT LINKED TO THE A117V MUTATION. Various amounts of VPSPr20, VPSPr17, and VPSPr7 were demonstrated by 1E4 in all 3 129 genotypes after deglycosylation and up to 50μg/ml of PK treatment (Fig 4A). Because the deglycosylation eliminated VPSPr26 and VPSPr23, these 2 fragments likely are the glycosylated isoforms of VPSPr20 and VPSPr17, respectively. This would explain the shared level of PK resistance of these 2 fragments (see Fig 3D). Of notice, the same PK-resistant fragments were well represented also without deglycosylation, suggesting that VPSPr20 and VPSPr17 are present as both glycosylated and unglycosylated isoforms (see Fig 3A, B). With 3F4, only VPSPr20 and VPSPr17 were detectable in the 129MM and 129MV cases, whereas again the 129VV genotype showed no PK-resistant PrP (see Fig 4B). The combined and individual resistance of the deglycosylated fragments to PK was comparable to that of the glycosylated isoforms.

Further characterization of the core fragments with the antibody anti-C (C-terminal residues 220–231) demonstrated 4 PrP bands migrating at approximately 20kDa, 18kDa, 12–13kDa, and 8kDa, of which only the 20kDa band matched VPSPr20 detected with 1E4 and 3F4 (see Fig 4C). The 3 fragments undetected by 1E4 and 3F4 must comprise the C-terminal region (reactive with anti-C) and must lack the 97–112 sequence

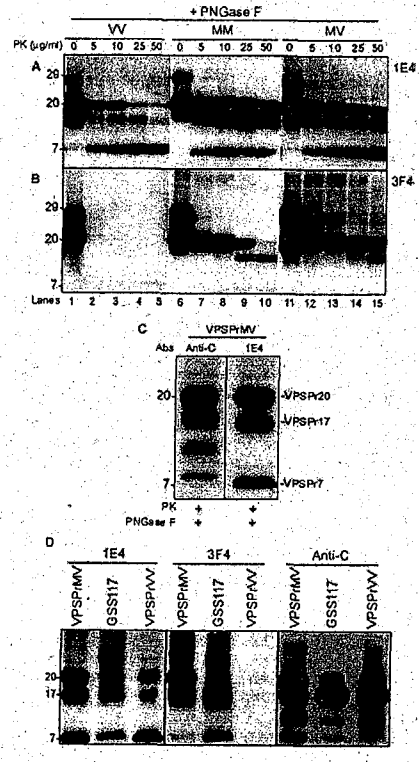

FIGURE 4: Proteinase K (PK)-resistant core fragments of variably protease-sensitive prionopathy (VPSPr) and their comparison with the disease-associated prion protein (PrP^{Dis}) fragments associated with Gerstmann-Sträussler-Scheinker disease (GSS)-A117V. *All brain homogenates were treated* with increasing concentrations of PK and with peptide N-glycosidase F (PNGase F). (A) With 1E4, all immunoblots from cases with the 129VV,129MM, and 129MV genotypes essentially show variably protease-sensitive prionopathy (VPSPr)-20, VPSPr17, and VPSPr7. However, the PK resistance of these bands varies according to the 129 genotype and the individual bands within the same genotype in a way roughly similar to that shown in Figure 3. (B) The immunoblots probed with 3F4 reveal 2 major bands in the 129MM and 129MV genotypes, which, however, exhibit a quite different pattern of resistance to PK in the 2 genotypes. As expected, no PK-resistant PrP bands are detected in the 129VV genotype. (C) Additional analysis of the core fragments following treatment with 25μg/ml of PK and PNGase, using the antibody anti-C to the PrP C-terminal residues 220–231. Compared to 1E4, anti-C demonstrates 4 bands of 20kDa, 18kDa, 12–13kDa, and 8kDa, respectively, of which only the 20kDa band has the same gel mobility as VPSPr20 detected with 1E4. The other 3 bands, including 18kDa, 12–13kDa, and 8kDa, do not match the bands detected with 1E4. (See text for explanation). (D) Brain homogenates from VPSPr-129MV, GSS117, and VPSPr-129 were treated with PK and PNGase F prior to Western blotting and probed with 1E4 *(left panel)*, 3F4 *(middle panel)*, and anti-C *(right panel)* antibodies. With monoclonal antibody 1E4, bands matching VPSPr20, VPSPr17, and VPSPr7 are detected in the GSS-A117V preparations, but the GSS-V117V bands immunoreact much less with 1E4 than the bands of VPSPr-129MV and VPSPr-129VV. The 23kDa band is seen more prominently in GSS-A117V. With 3F4, the VPSPr17 and VPSPr7 bands are shared by GSS-A117V and VPSPr-129MV, but the 20kDa band is missing in GSS-A117V. The VPSPr7 band is much less reactive in 129MV. As previously, the 129VV is not reactive with 3F4. Anti-C reveals apparently the same bands in all 3 preparations, but with significantly different ratios. M = methionine; V = valine.

containing the 1E4 and 3F4 epitopes. Therefore, 6 core fragments of relative molecular weights between 20kDa and 7kDa were identified by the combined use of 1E4 and anti-C. Several PrP C-terminal fragments of similar relative molecular weight have been previously reported.[18–20] Considerable similarities were observed in the electrophoretic mobilities of the PK-resistant core fragments from the 129MV, 129VV, and 129MM genotypes and GSS-A117V (see Fig 4D). Also in GSS-A117V, the mAb 1E4 demonstrated the presence of 3 bands of 20kDa, 17kDa, and 7kDa described in VPSPr, which, however, displayed different immunoreactivities. Comparable variations in antibody immunoreactivity and band representation were seen with 3F4 and anti-C (see Fig 4D). Therefore, most of the PK-resistant PrP^{Dis} fragments appeared to have similar sizes in VPSPr and GSS-A117V, but different ratios and antibody reactivities.

Discussion

At variance with the series of 11 cases of PSPr described in 2008 and 2 cases subsequently published by others—all 13 of which were 129VV homozygous at the PrP gene—the 15 cases reported here also include affected subjects who are 129MV and 129MM, in addition to new 129VV subjects.[3–5] Comparative analyses indicated that all these cases are affected by the same disease process, and that most of the heterogeneity that we observed results from distinct 129 genotypes.

These cases are likely to be affected by the same disease because of the overall similarity in major phenotypical characteristics, including the clinical features, which prominently exhibit aphasia, ataxia, and parkinsonian signs; SD, displaying vacuoles comparable in size in the 3 genotypes but otherwise different from the vacuoles of other common prion diseases; and finally PrP immunostaining patterns, which also display comparable general features in all these cases. However, the 2 most striking similarities reside in the ladderlike electrophoretic pattern of the PK-resistant fragments and in the unique immunoreactivity of PrP^{Dis} with mAb 1E4. Cumulatively, these findings suggest that all these cases share a similar molecular mechanism of PrP^{Dis} formation.

Significant clinical differences among the 129VV and 129MV groups (only 2 129MM symptomatic subjects were available) occurred in the mean age at onset and in disease duration (see Table). PrP immunostaining patterns were also distinguishable in the 3 groups. An additional difference might lay in disease prevalence, which appeared to be highest in 129VV subjects (65% of the cases), followed by the 129MV (23%) and 129MM subjects (12%) (see Table). However, 2 distinctive features were evident among the 3 groups; these were: (1) the apparent resistance to PK digestion, which was generally much lower in the 129VV cases than in the 129MM and 129MV cases; and (2) the immunoreactivity of the PK-resistant PrP^{Dis} with mAb 3F4, which was strong in the 129MM cases, weak in the 129MV cases, and lacking in the 129VV cases. Cumulatively, these findings argue that, although PrP^{Dis} may be formed by a similar mechanism in the 3 genotypes, the conformation or aggregation is likely different, and this difference results in variable resistance to PK, variable accessibility by 3F4, or both.

These findings also indicate that, in the present series of cases, it is the 129 genotype that modifies the phenotypic characteristics, including PK resistance and antibody immunoreactivity of PrP^{Dis}. However, the possibility that phenotypic heterogeneity is caused by other variations in PrP^{Dis} among the 3 groups or by a combination of different 129 genotypes and PrP^{Dis} characteristics, as is the case with sCJD, cannot be excluded.

The variations in prevalence are likely to be associated with the 129 genotype as well. This also is a feature of sCJD, in which 129MM cases account for about 70% of the total, 129MV for 11%, and 129VV for 17%.[7,12] It is remarkable that the effect of the 129 genotype on disease prevalence in our series of cases appears to be the opposite of that in sCJD. The high percentage of 129VV subjects described to date (20 of 28 known subjects, including the 2 cases reported elsewhere) and the apparent rarity of 129MM subjects (only 3 of 28 subjects) suggest that the prevalence of VPSPr is directly related to the presence of the 129V allele.[3–5] Indeed, at least 1 129V allele is present in 25 of the 28 known cases of VPSPr. The prevalence of the 3 129 genotypes in VPSPr is quite different from that in normal Caucasian populations, in which the 129MM genotype accounts for 43% of subjects, the 129MV for 49%, and the 129VV for 8%.[21]

The present findings raise a number of questions concerning the nature of VPSPr and its place within the group of known prion diseases.

In our series of 26 VPSPr cases collected to date, 8 subjects apparently had familial dementia; they were all 129VV except for 1 129MM. One of the 2 VPSPr-129VV cases reported by others also had a definitive family history of neurodegenerative disease.[4] This raises the possibility that VPSPr is a familial disease with a locus other than the ORF of the PrP gene (which is free of mutations), a condition analogous to that of familial Alzheimer disease.[3,22] Whether the VPSPr subjects reported to date also include inherited cases belonging solely to the 129VV and 129MM genotype remains to be determined.

Well-recognized prion diseases, which are associated with the classic PrP^{Dis} commonly identified as PrP27-30, such as all sCJD subtypes and several subtypes of familial CJD and sporadic and familial fatal insomnia, are transmissible with relative ease to receptive animals.

Inoculated animals develop a full-blown disease with clinical signs, SD, and presence of a PrP27-30 that generally reproduces the characteristics of the PrP27-30 present in the inoculum.[1,23,24] In contrast, other prion diseases, especially GSS, a rare phenotype that to date has been reported as exclusively associated with PrP gene mutations, have been more difficult to transmit or have been reported not to be transmissible at all.[25–28] For example, inoculation of brain homogenate from a subtype of GSS linked to the P102L mutation and characterized by the immunoblot presence of only a PK-resistant fragment of 7kDa similar to that present in VPSPr did not cause a symptomatic disease in recipient transgenic mice, but elicited the formation of PrP amyloid deposits in the absence of abnormal PrP.[26] Similarly, inoculation of PK-sensitive recombinant PrP polymerized into amyloid fibers generated a prion disease in PrP overexpressing transgenic mice that was apparently asymptomatic but caused SD and deposition of PK-sensitive abnormal PrP. 2 features shared by the 129VV genotype of VPSPr, only late in the life of the inoculated animals, consistent with very long incubation times.[29] Furthermore, similar transmission patterns on inoculation of brain homogenates from affected animals or humans have been observed in other neurodegenerative diseases, such as Alzheimer disease and diseases of the tau protein or tauopathies.[30,31] Experiments on the transmissibility of VPSPr are ongoing. Preliminary data indicate that VPSPr transmissibility, if it occurs at all, is not efficient, and it could be more like that of GSS-P102L associated with PrP^{Dis} 7kDa or of PK-sensitive PrP amyloid fibers, which require long incubation times and do not shorten the life span of the affected animals.[26,29]

It is intriguing that GSS also shows characteristics of the phenotype and of the PrP^{Dis} associated with some of the mutations that resemble those of VPSPr.[25] They include long disease duration, multiple PK-resistant fragments, and variable PK resistance of PrP^{Dis}. Our comparative analysis of the electrophoretic profiles in VPSPr and GSS-A117V reveals provocative similarities. This finding raise the issue of whether VPSPr might be viewed as the sporadic form of GSS.

Regardless of its relationship with GSS, the finding that VPSPr affects all 3 129 genotypes, resulting in distinct disease phenotypes and PrP^{Dis} characteristics, establishes VPSPr as the second "sporadic" prion protein disease, after sCJD.

Acknowledgment

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Potential Conflicts of Interest

Nothing to report.

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130 研究報告の概要

この文書は、2003 年 2 月に公表され、2004 年 6 月と 2010 年 XXX に改訂された「クロイツフェルト-ヤコブ病と、血漿由来医薬品および尿由来医薬品」についての CPMP(脚注 2)の見解表明書(position statement) (EMEA/CPMP/BWP/2879/-02)の第 2 改訂版であり、1998 年 2 月に公表された「新しい変異型 CJD と血漿由来医薬品」についての CPMP の見解表明書に置き換わるものである。

要約

疫学的なエビデンスが集積してきたが、それらのエビデンスは血漿由来医薬品によってクロイツフェルト-ヤコブ病(CJD)の伝播が起こるとの考え方を裏付けるものではない。あるドナーがドネーション後に孤発性、家族性、もしくは医原性 CJD であることが確認された場合に、血漿由来医薬品のリコールを求めることは妥当ではない、とのこれまでの CHMP の立場には変化はない。

変異型 CJD(vCJD)は新たに出現してきた疾患であり、症例数が最終的にどの程度となるのかは不確かである。vCJD は孤発性 CJD と比較すると末梢組織中への分布がより広く感染性/異常プリオンタンパク質のレベルがより高い。英国で輸血によるヒトへの医原性 vCJD 感染が明確な 4 例から vCJD が輸血を介して伝播しうるものである、ということの強力なエビデンスが得られた。2009 年に、中程度の純度の第 VIII 因子の投与を受けた血友病 A 患者で感染性物質が検出されたが。この製剤は英国で 1998 年より前に採取されたプール血漿から調製されたものであった。

英国に居住していることは vCJD の危険因子とされており、そのことから英国は英国起原の血漿から分画を今後行わないことを決定するに至った。危険期間中に英国に長期間滞在したドナーから血液/分画用血漿のドネーションを受けないようにすることはこの英国の決定と一貫した措置である。1980 年初頭から 1996 年末までの間に英国に累積で 1 年間以上滞在したドナーを、血液/分画用血漿のドネーションから排除することを勧告する。

あるドナーについて英国での滞在に基づけば排除すべきであったことがドネーション後に判明した場合に、当該バッチを回収することは勧告されていないが、それは、このような排除が非常に慎重を期した予防措置だからである。

現在得られているデータでは、ヒト血漿中に vCJD の感染性が仮に存在していたとしても、血漿由来医薬品の製造工程で vCJD の感染性は低減されることが示されている。製造者にはその製造者の用いている製造工程で感染性を低減させる能力がどの程度あるか、ステップごとに調べて推定することが求められている。製造者がこの推定を行う際には、マイルストーンの各々でこの分野の専門家と相談することを勧告する。CHMP とそのバイオテクノロジー作業部会(BWP:Biotechnology Working Party)は、これらの勧告事項と取るべきアクションについて検討を続けるつもりである。

この勧告事項のサポートとして、CHMP と BWP は、外部の専門家の関与も依頼して、vCJD の危険性に関して製造工程の調査方法に関するガイダンスを作成したが、CHMP と BWP は生ずる可能性のある問題点について討議する用意がある。

このような立場を取る理由は、仮に将来、血液および血漿由来医薬品製造のための血漿を採取し集めている国々においてさらに vCJD の症例が発生したならば、以前に TSE 感染性を低減しうることが示されていた製造工程は、過去の製剤の安全性の再保証を与えることとなり、分画を継続することを妥当とすることの助けとなろう。

スクレイピーに感染したげっ歯類の尿中、および慢性消耗性疾患のシカの尿中に低レベルの TSE 感染性物質が検出されている。しかし、尿由来の医薬品による CJD もしくは vCJD の伝播の疫学的証拠はない。尿由来医薬品の製造工程の総合的レビューでは、ある製剤が比較

使用上の注意記載状況・ その他参考事項等

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医薬品
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的少数で特徴のよく分かっているドナー集団由来のものである場合には、ドナー選択基準を適用することが可能であることを指摘している。さらに、そのレビューでは、TSE感染性が出発材料中に存在していたとした場合でも、理論的にはその感染性を低減することができると考えられるステップを、少なくとも1つ製造工程中に有していることを指摘している。尿由来医薬品は英国で採取された尿を原料とはしないこととなっている。 このレビューおよびその他の考慮事項に基づいて、可能であれば、尿ドナーパネルを選択するための除外基準の使用が、予防的措置として勧められる。血漿由来医薬品の製造のための出発材料を提供する血液/血漿ドナーに用いるものとしてCJDおよびvCJDに関しても同じ除外基準が適用されるべきであるが、血液/血漿ドナーとは異なり、それらの判定基準は各ドネーションごとにはチェックされない。尿由来医薬品の製造者が、血漿由来医薬品のためのアプローチと類似のものに従って、製造工程のTSE感染性物質の低減/排除能を評価することを勧告する。	2. 重要な基本的注意 (1)略 1)略 2)略 3)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。

報告企業の意見	今後の対応
これは血漿由来と尿由来製品（EMEA/CPMP/BWP/2879/02)のクロイツフェルト-ヤニブ病に関するCPMPの見解表明書の第2改訂(案）である。 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、弊社の原料血漿採取国である日本及び米国では、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、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研究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るものではないと考えるので、特段の措置はとらない。

コンコエイト

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

1 London, 24 June 2010
2 EMEA/CPMP/BWP/2879/02/rev 2
3 Committee for Medicinal Products for Human Use (CHMP)

4 # CHMP position statement on Creutzfeldt-Jakob disease and plasma-derived and urine-derived medicinal products

5 and plasma-derived and urine-derived medicinal products
6 Draft[1]

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7 Comments should be provided using this template. The completed comments form should be sent to Alberto.Ganan@ema.europa.eu

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Keywords	Creutzfeldt-Jacob disease, human Transmissible Spongiform Encephalopathies, plasma-derived medicinal products, urine-derived medicinal products, sporadic CJD, genetic CJD, iatrogenic CJD, variant CJD, blood infectivity, transmissibility

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[1] Delete once the reflection paper is adopted.

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CHMP position statement on Creutzfeldt-Jakob disease and plasma-derived and urine-derived medicinal products

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This is the second revision of the CPMP[2] Position Statement on "Creutzfeldt-Jakob disease and plasma-derived and urine-derived medicinal products" (EMEA/CPMP/BWP/2879/02) published in February 2003 and revised in June 2004 and XXX 2010, which replaced the CPMP Position Statement on "New variant CJD and plasma-derived medicinal products" (CPMP/201/98) issued in February 1998.

Summary

Cumulative epidemiological evidence does not support transmission of sporadic, familial and iatrogenic Creutzfeldt-Jakob disease (CJD) by plasma-derived medicinal products. There is no change to the previous CHMP position that recall of plasma-derived medicinal products is not justified where a donor is later confirmed as having sporadic, familial or iatrogenic CJD.

Variant CJD (vCJD) is an emerging disease and the eventual number of cases of the disease is uncertain. There is a wider distribution and higher level of infectivity/abnormal prion protein in peripheral tissues than is seen with sporadic CJD. Four instances of apparent iatrogenic vCJD infection by blood transfusion in man in the UK provide strong evidence that vCJD is transmissible through blood transfusion. In 2009, the agent was detected in a haemophilia A patient who received intermediate purity FVIII prepared from pooled plasma sourced in the UK before 1998.

Residence in the UK is a recognised risk factor for vCJD and has led to the UK deciding to no longer fractionate from UK plasma. It is consistent with this decision to exclude donors who have spent long periods in the UK during the risk period from donating blood/plasma for fractionation. It is recommended that donors who have spent a cumulative period of 1 year or more in the UK between the beginning of 1980 and the end of 1996 are excluded from donating blood/plasma for fractionation.

There is no recommendation to recall batches if information that would have excluded a donor based on his/her stay in the UK becomes available post-donation, since this is a very conservative precautionary measure.

Available data indicate that the manufacturing processes for plasma-derived medicinal products would reduce vCJD infectivity if it were present in human plasma. Manufacturers are required to estimate the potential of their specific manufacturing processes to reduce infectivity using a step-wise approach. It is recommended that manufacturers consult the relevant competent authorities at each of the milestones in this estimation. CHMP and its Biotechnology Working Party (BWP) will keep progress with these recommendations and the actions to be taken under review.

In support of this recommendation, CHMP and BWP, with the involvement of external experts, have developed guidance on how to investigate manufacturing processes with regard to vCJD risk and CHMP and BWP are available to discuss issues that might arise.

The rationale for this position is that if, in the future, further cases of vCJD occur in countries collecting blood and plasma for the manufacture of plasma-derived medicinal products, a process previously shown to be able to reduce TSE infectivity will provide reassurance on the safety of past products, and could help to justify continuing fractionation.

Low levels of infectious TSE agents have been detected in the urine of scrapie-infected rodents and in the urine of deer with Chronic Wasting Disease. However, there is no epidemiological evidence of CJD or vCJD transmission by urine derived medicinal products. A general review of manufacturing

[2] In May 2004 there was a change in the name of the EMEA's scientific committee for human medicines from CPMP to CHMP.

processes for urine-derived medicinal products indicates that it is feasible to apply donor selection criteria when a product is derived from a relatively small and well-defined donor population. In addition, it indicates that manufacturing processes have at least one step that might be theoretically capable of reducing TSE infectivity if it were present in the starting material. It is noted that urine-derived medicinal products are not sourced from urine collected in the UK.

On the basis of this review and other considerations, the use of exclusion criteria for selection for a urine donor panel is encouraged, as a precautionary measure, where feasible. The same exclusion criteria should be applied with respect to CJD and vCJD as used for blood/plasma donors providing starting material for the manufacture of plasma-derived medicinal products but, unlike blood/plasma donors, these criteria would not be checked at each donation. Manufacturers of urine-derived medicinal products are recommended to evaluate the capacity of the manufacturing process to reduce/eliminate TSE agents by following a similar approach to that for plasma-derived medicinal products.

1. Introduction

Creutzfeldt-Jakob disease (CJD) is a rare neurodegenerative disease belonging to the group of human Transmissible Spongiform Encephalopathies (TSEs) or prion diseases. Mortality rate of TSEs ranges approximately from 1.5 to 2 persons per million population per year. TSEs can occur sporadically (sporadic CJD (sCJD) and sporadic fatal insomnia), be associated with mutations of the prion protein gene (genetic TSEs (gTSE)), or result from medical exposure to infectious material (iatrogenic CJD (iCJD)). In 1996, a variant form of CJD (vCJD) was identified.[1] There is strong evidence that vCJD is caused by the agent responsible for bovine spongiform encephalopathy (BSE) in cattle.[2,3,4] The most likely hypothesis is that vCJD has occurred through exposure to BSE contaminated food.

Human TSEs, including in particular vCJD, were addressed in expert meetings/workshops at the EMEA in January 1998, January 1999, December 1999, May 2000, and December 2000. A CPMP Position Statement on variant CJD and plasma-derived medicinal products was issued in February 1998[5f] and the outcome of the subsequent meetings was published on the EMEA website.[5] An EMEA Expert Workshop on Human TSEs and Medicinal Products was held on 19-21 June 2002. This provided the scientific basis for a new CPMP Position Statement issued in 2003.[5b] A further EMEA Expert Workshop was held in January 2004 to review the current state of knowledge of vCJD, in the light of the recent report of a possible human transmission by blood transfusion.[6] In addition, the Workshop discussed the CPMP Discussion document on the investigation of manufacturing processes with respect to vCJD.[5a] In October 2005, a follow-up workshop was held to discuss the number of vCJD cases reported in France and other European countries and the potential effect of additional donor exclusion measures. Urine-derived medicinal products were specifically discussed at an EMEA expert workshop in July 2007[5g] after publication of experiments indicating transmission of prions via urine using a hamster model.

Blood and blood components for transfusion are outside the scope of this Position Statement. Recommendations on the suitability of blood and plasma donors and the screening of donated blood in the European Community were described in Council Recommendation 98/463/EC.[7c] European legislation on human blood and blood components entered into force on 8 February 2003[7a] Under this legislation, a Commission Directive on certain technical requirements for blood and blood components, including eligibility criteria for donors, entered into force in April 2004.[7b] In addition, Council of Europe Recommendation No. R (95) 15 contains a technical appendix on the use, preparation and quality assurance of blood components and details the current requirements for donors.[7g]

In December 2003, following the announcement of a possible case of vCJD transmission by blood transfusion, Commissioner Byrne made a statement highlighting EU activities in the area of vCJD and announcing a meeting of the Working Group of the Blood Regulatory Committee to consider the latest information available from the UK.[7d] The meeting took place in January 2004 and a summary statement was produced.[7e]

The Scientific Steering Committee (SSC) and the Scientific Committee on Medicinal Products and Medical Devices (SCMPMD) of the European Commission have published a number of opinions relating to TSEs, which are of relevance to blood and blood components for transfusion, as well as to plasma-derived medicinal products.[8] WHO Guidelines on TSEs are also of relevance to both blood components for transfusion and plasma-derived medicinal products.[9] The Council of Europe has made recommendations for blood and blood components for transfusion.[10]

2. Human TSEs current status

2.1. Sporadic, genetic and iatrogenic forms of human TSEs

There is no evidence that sporadic, genetic or iatrogenic forms of human TSEs have been transmitted from person to person through exposure to plasma products or urinary derived medicinal products. Systematic surveillance for CJD of all types has been undertaken in a number of countries, including a collaborative study in the EU since 1993,[11,12] and no case of sporadic, genetic or iatrogenic CJD has been causally linked to prior treatment with plasma products. Cases of sporadic CJD with a history of drug treatment for infertility have not been identified but there is uncertainty about the validity of this observation. (See the report of the 2007 EMA expert meeting for further details.[5g]) Although there is evidence that plasma products have not been implicated in transmission of sporadic, genetic or iatrogenic CJD, the strength of the evidence excluding transmission by urinary derived medicinal products is less secure.

2.2. Variant CJD

The official UK figures for vCJD at the beginning of April 2010 were a total of 172 definite or probable vCJD cases.[13] (One case diagnosed in Hong Kong was classified as a UK case and is included in the UK figures.) Outside of the UK, there have been 25 cases in France[15], 5 in Spain, 4 in the Republic of Ireland, 3 in the Netherlands, 3 in the USA, 2 in Portugal and Italy and single cases in Canada, Saudi Arabia and Japan. 2 of the Irish cases, 2 of the US cases, 1 French case and the Canadian case had spent more than 6 months in the UK during the period 1980-1996 and were probably infected while in the UK. [14] The third US case has been reported as most likely infected when living in Saudi Arabia. The possibility of cases occurring in other countries cannot be excluded.

Two cases of vCJD identified in Spain occurred in the same family. No family links have been reported in any other vCJD cases to date.

All definite and probable cases, which have been genotyped so far, are Met-Met homozygotes at codon 129 of the prion protein (PrP) gene.[16] In 2009 a possible case of variant CJD was reported in the UK with a heterozygous codon 129 genotype. [17]

Analysis of the UK figures for the quarterly incidence of deaths indicates that vCJD incidence in the UK is currently in decline. However, interpretation requires caution as there may be a long tail or more than one peak to the epidemic.[18]

A UK study screening specimens from surgically removed appendices and tonsils for accumulation of prion protein in the lymphoreticular system has been carried out in order to try and obtain some estimation of the number of people that might be incubating vCJD in the UK.[19] Three positive appendix specimens have been found as a result of the screening of 12,674 appendix and tonsil specimens. However, the pattern of lymphoreticular accumulation in two of these samples was dissimilar from that seen in known cases of vCJD, raising the possibility that they may be false positives. With respect to this possibility, the authors comment that although it is uncertain whether immunohistochemical accumulation of prion protein in the lymphoreticular system is specific for vCJD, it has not been described in any other disease, including other forms of human prion disease or a range of inflammatory and infective conditions. Subsequent genetic analysis of residual tissue samples from these 2 cases found that both were valine homozygotes at codon 129 in the prion protein gene[20] This finding might account for the immunohistochemical features in these cases; all patients who have developed vCJD and have undergone a comparable genetic analysis have been methionine homozygotes at codon 129 in the prion protein gene.

Statistical analysis on this finding of 3 positive specimens gives the following estimations of numbers who may be incubating vCJD:

237 infections per million population (95% confidence interval (CI): 49-692 per million)

Assuming that this estimate relates to those aged 10-30 years[3], 3,808 individuals (CI 785-11 128) aged 10-30 years may be incubating vCJD in the UK.

These estimations are higher than predictions from modelling of the clinical data (upper 95% confidence interval of 540 future cases).[21] It is not known whether those incubating vCJD will eventually develop clinical disease. However, estimates of numbers possibly incubating are important with respect to any potential for secondary transmission (e.g. by blood donation, surgical instruments) while individuals are in the incubation phase. It should be noted that plasma-derived medicinal products have not been manufactured from donations collected in the UK since 1998.

A larger study of an archive of tonsil tissue from 63,007 people of all ages removed during routine tonsillectomies has been published.[22] 2,753 samples were from the 1961- 1985 birth cohort in which most cases of vCJD have arisen and 19,808 were from the 1986-1995 birth cohort that may also have been orally exposed to bovine spongiform encephalopathy. None of the samples were unequivocally reactive to two enzyme immunoassays and none of the initial reactives were positive for PrP^{TSE} by immunohistochemistry or immunoblotting. The estimated 95% confidence interval for the prevalence of PrP^{TSE} in the 1961-1995 birth cohort was 0-113 per million and in the 1961-1985 birth cohort 0-289 per million. These estimates are lower than the previous study of appendix tissue, but are still consistent with this study. Archiving of tonsil tissues continues and further studies are planned.

3. Human tissue distribution of infectivity/abnormal prion protein.

Tissue distribution has been investigated by detection of the abnormal prion protein PrP^{TSE} or by infectivity assays. Detection of PrP^{TSE} in tissues has often been associated with infectivity, however it should be noted that, in some circumstances, infectivity can be present without detection of PrP^{TSE} or PrP^{TSE} be present in absence of infectivity.[23] This may be related to limitations of assay methods for PrP^{TSE}, however, in some cases the reason for this finding is not known. It is thus recommended that any study on tissue or fluid distribution of the abnormal prion protein be confirmed with an infectivity assay.

A wider distribution and higher level of PrP^{TSE} in human peripheral tissues, including the lymphoreticular system, has been found in vCJD compared with sporadic CJD.[24;25;26] Limited data from infectivity assays of vCJD tissues are consistent with the PrP^{TSE} findings.[27] In clinical vCJD cases high titres of infectivity are found in the brain and spinal cord and lower levels in spleen and tonsil[27]. While PrP^{TSE} and infectivity are occasionally found in the spleen of sporadic CJD, the levels of PrP^{TSE} are lower than in vCJD.[81] It is also suspected that lymphoid tissue involvement in sCJD is associated with a relatively long duration of clinical illness whereas it occurs preclinically in vCJD. PrP^{TSE} accumulations have been observed in muscles of some patients with both sporadic and variant CJD.[28]

It is likely that the distribution of PrP^{TSE} and infectivity in iCJD is more similar to sCJD than vCJD.[29] Data are lacking for gCJD.

4. Infectivity in blood and transmissibility via blood

4.1. Animal blood

Low levels of infectivity have been found in the blood of rodents experimentally infected with animal and human TSE agents.[30,31,32,33] Experiments indicate that approximately half the infectivity is in the cellular components, mainly the buffy coat, and the remainder in the plasma. Experimental studies indicate that the vCJD agent behaves in a similar way (qualitatively and quantitatively) to a genetic TSE agent[4] when adapted to RIII/Fa/Dk mice.[33] Infectivity has also been detected in buffy coat of a prosimian microcebe experimentally infected with a macaque-adapted BSE strain.[34]

The infectivity in rodent blood was transmitted by intravenous inoculation, but 5-7 fold less efficiently than by the intracerebral route.[31] In one study with mouse-adapted vCJD agent, the intravenous and intracerebral routes were found to be equally efficient for the buffy coat fraction but not for the plasma fraction.[33] However, studies in primates show that survival times were similar after intravenous or intracerebral inoculation of infected brain material.[35,36] Unpublished studies presented at scientific meetings[37,38] indicate that blood of primates experimentally infected with human TSE agent is infectious from about half way through the incubation period.

Furthermore, information from intra-species transfusion experiments indicates that experimental BSE in orally infected sheep or natural scrapie infection in sheep can be transmitted to sheep by blood transfusion.[39,40] Transmission efficiency was high for both BSE and natural scrapie, and the majority of transmissions resulted from blood collected more than half way through the incubation period[41]. The level of infectivity in sheep blood cannot be established from these experiments.

The European Union has provided funding for animal transmission projects.

4.2. Human blood

The tracing of recipients of blood transfusion from UK donors who have subsequently developed vCJD (the TMER study) has revealed four instances of secondary transmission.[42] These individuals had received transfusion of non-leucodepleted red cells from donors who were clinically healthy at the time of donation but subsequently (17–40 months later) developed variant CJD. Three of the four patients developed disease after incubation periods ranging from 6.5 to 8.5 years; the fourth died 5 years after

[3] The reason the age range of 10-30 years is specified is because 83% of the samples were from individuals in this age range.

[4] Mouse-adapted GSS strain of human TSE (brain tissue obtained from a case of Gerstmann-Sträussler-Scheinker syndrome).

transfusion of an illness unrelated to prion disease but tested positive for PrP^{TSE} in the spleen and lymph nodes. This asymptomatic prion-infected patient was heterozygous (methionine/valine) at codon 129 of the *PRNP* gene. Taken together, these instances are strong evidence that vCJD is transmissible through blood transfusion.

Recently, another presumed case of prion infection was identified in an elderly haemophilic patient who was heterozygous at codon 129 in the prion protein gene.[43] The patient, who died of unrelated pathology, had received large quantities of UK-sourced fractionated plasma products, including some units derived from plasma pools which contained plasma from a donor who later developed variant CJD. This patient was identified through an intensive search for PrP^{TSE} positivity in all post-mortem tissues, although only 1 of 24 samples taken from the spleen tested positive. Whether someone with this limited distribution of PrP^{TSE} would be infectious is unknown, but from a public health perspective, this patient represents a warning that some plasma-derived products might contain residual prion infectivity.

The surveillance described above emphasises the importance of the TMER study for identifying the risk of blood transfusion in transmitting vCJD. Moreover, national databases of blood donors and the maintenance of traceability from donor to recipient and vice versa are essential to establish whether a vCJD case has been a blood donor (UK experience has shown that questioning of family members is unreliable for establishing whether a patient has been a blood donor). Traceability is a specific requirement in Article 14 of Directive 2002/98/EC.[7a]

Infectivity or PrP^{TSE} were not detected in blood of vCJD cases using methods capable of detecting infectivity/PrP^{TSE} in peripheral tissues such as tonsil or spleen, indicating that if infectivity is present it is at levels below the sensitivity of these methods. [27,24]

There is no epidemiological evidence that blood of sporadic CJD may transmit disease.[44,45] Prospective studies, similar to the TMER study, are in progress in the UK and USA and have not yet revealed any possible case of sporadic CJD linked to blood transfusion. However, current data are scanty to unequivocally exclude the possibility that such an event could occur in a small number of cases with a long (10 or more years) incubation period.[46]

A review of transmission studies to detect infectivity in the blood of humans with CJD (sporadic, iatrogenic and variant) shows that although experimental transmissions to animal models have occasionally been reported[47-50], other studies failed to detect infectivity.[51,27] It remains possible that PrP^{TSE} is present at low levels in the blood of clinically affected cases of sCJD. Data are lacking for gCJD but the assumption is that the tissue distribution of infectivity will be more similar to sCJD than vCJD.

For the purpose of risk assessments, it is recommended that, as a worst case assumption, a relative efficiency of the intravenous and intracerebral routes of 1:1 should be used.[52] This is because the accumulated information now available from animal studies indicates that the intravenous route can be an efficient route of transmission and in certain cases can give a transmission rate and/or an incubation period similar to the intracerebral route (see also 4.1).

5. Detection techniques

Several techniques are under development for the detection of PrP^{TSE} in blood including methods based on epitope protection[53] and PrP^{TSE} specific antibodies[54]. Approaches based on surrogate markers are also under investigation. Development and validation of all methods is on-going but there is no screening test yet. Confirmatory tests that have been proposed include Protein Mis-folding Cyclic Amplification (PMCA) [55] which is extremely sensitive, but has not yet been validated.

Several WHO reference preparations are available and further materials are under development[9b]. These reference preparations will allow calibration of assays versus infectivity bioassays, and can be used for collaborative studies to compare the performance of different assays to see whether they are sufficiently sensitive and specific to justify further evaluation for screening blood.

PrP^{TSE} detection methods for screening human blood for evidence of infection are being considered for inclusion as Annex II List A devices under the IVD Directive. There are very few samples of blood or plasma from clinically affected patients or from individuals known to have been infected at a particular time. This contrasts with other blood borne agents such as viruses. Alternative development and evaluation strategies have been proposed to assess whether a candidate assay is sufficiently promising to be given access to the available samples.[56]

6. Leucoreduction and specific prion affinity filters

Leucoreduction is used in transfusion medicine to reduce the level of white blood cells in blood and blood components. It was implemented in the UK in 1999.

The rationale for considering leucoreduction as a precautionary measure is:

- The lymphoreticular involvement in vCJD
- The detection of low levels of infectivity, in studies with rodents, in the buffy coat (associated with white blood cells).

The SCMPMD opinion on leucoreduction [8a, 8b] for blood and blood components for transfusion states that it might be a precautionary step to remove white cells as completely as possible. For plasma for fractionation the opinion states the following:

'Taken together, there is no compelling scientific evidence to date for the introduction of leucoreduction of plasma for fractionation, or other methods aiming at removal of cells and debris, as a precaution against vCJD transmission. The question should be further explored by suitable experiments.'

Results reported at the 2002 EMEA Workshop, suggested that leucoreduction does not provoke fragmentation of cells and lysis. Results of a comprehensive study involving a number of different filters and procedures indicate that leucodepletion is not detrimental in terms of the generation of microvesicles or the release of prion proteins[57].

Infectivity data from hamster studies indicate that leucoreduction alone is not totally protective against prion transmission, with between 42 to 72 percent reduction in infectivity of whole blood[58,59].

Specific affinity ligands that bind prion proteins are being evaluated for their ability to reduce TSE infectivity present in blood and plasma.

A study in hamsters showed that a leucocyte-reduction filter based on modified polyester fibres exhibited a prion clearance capability between 99.0 to 99.9 percent on the endogenous and exogenous infectivity of red cell concentrates[60].

Initial studies using leucoreduced human red blood cell concentrates spiked with hamster brain-derived scrapie infectivity indicate that some ligands immobilised on a chromatographic resin matrix are capable to removing 3 to 4 log ID_{50} per ml[59]. A further study using scrapie-infected hamster whole blood demonstrated an overall reduction of infectivity of more than 1.22 log ID[61].

The prion binding capacity of an affinity ligand chromatography step has been investigated in the processing of a plasma medicinal product using hamster brain derived spiking material[62]. This preliminary data requires further evaluation before conclusions can be drawn on possible efficacy.

7. Manufacturing processes for plasma-derived medicinal products

Taking account of the available data concerning blood infectivity, it is of utmost importance to investigate the capacities of the manufacturing process (fractionation) to eliminate/inactivate the infectious material potentially present in the plasma pool used as the starting material for preparation of plasma-derived products. Initial results from animal studies, using blood from rodents infected by intracerebral inoculation, indicated that the fractionation process contributes to the removal of endogenous plasma infectivity.[30,31] Information reported at the EMEA Workshops in 2002 and 2004 suggested that endogenous infectivity might persist through the fractionation process to a greater extent than would be expected from spiking studies,

Many investigational studies have now been carried out with different strains of agent and spiking materials of different nature and purity, and using different assays to follow the partition of PrP^{TSE} and/or infectivity. In most cases, the correlation between the capacity to partition PrP^{TSE} and infectivity has been demonstrated for the spiking preparations used until now (mainly brain homogenates of various strains). It is now confirmed that biochemical assays can be useful for spiking experiments to investigate manufacturing processes in a reasonable timeframe and less costly protocols than the *in vivo* bioassay. However it is still necessary to correlate such results with those from infectivity assays in animals. Cell-based assays may also be useful if properly validated for this purpose.

Studies aimed at investigating the contribution of the various manufacturing steps to reduction of infectivity (including precipitation followed by centrifugation or depth filtration, chromatography and nanofiltration) have accumulated convergent data supporting the removal of infectivity by steps that are commonly used in the manufacture of plasma-derived medicinal products.[62-68] For coagulation factors derived from cryoprecipitate, downstream fractionation using various precipitating agents or conditions allow to discard PrP^{TSE} in the precipitates. Reduction level achieved may vary according to the specific manufacturing process and probably depends on the concentration of the precipitating agent and salts, and the pH. Chromatographic steps, classically used in the separation of coagulation factors but also in the purification of other plasma derivatives have been described to remove TSE infectivity or PrP^{TSE}. Again, the reduction factors may be variable according to the fraction eluted. However, caution is still needed in the interpretation of those data since the effectiveness of a given step is dependent on a number of variables including the process conditions and the state/nature of the agent in the spiking preparation sample and in the spiked product intermediate. Consequently, effectiveness of removal may vary from one manufacturer to another. In addition, recent studies have highlighted the fact that removal capacity may be variable according to the state of dispersion of the agent in the spiking preparation particularly for steps based on retention mechanisms.

Overall, there is a need i) to investigate the partitioning or removal capacities of the various fractionation steps used in the preparation of the plasma-derived medicinal products, ii) to investigate the partition and removal of endogenous infectivity and the extent to which this is comparable with data from spiking studies, iii) to gain better knowledge of the form of infectivity present in blood in order to confirm the relevance of the spiking material used in the validation studies.

8. Infectivity in urine

Low levels of infectivity have been detected in urine of scrapie-infected rodents by several research groups and in the urine of deer with Chronic Wasting Disease.[5g, 9c]

Gregory *et al.*[69] demonstrated that the disease could be transmitted by intracerebral inoculation of pooled urine from scrapie-sick hamsters. The infectivity titre of the urine was calculated to be around 3.8 infectious doses/ml. Titration of kidney and urinary bladders from the same animals gave 20,000-fold greater concentrations. Histologic and immunhistochemical examination of these tissues showed no indication of inflammation or other pathologic changes, except for occasional deposits of disease-associated prion protein in kidneys.

Kariv-Inbal *et al.*[70] have observed transmission of the disease after intraperitoneal (i.p.) administration of enriched urine fractions from scrapie sick hamsters. Transmission via the oral route was also investigated. The recipient hamsters remained without symptoms but secondary transmission was observed after inoculation of brain extract from an asymptomatic hamster.

Seeger *et al.*[71] have studied transmission via urine using mouse models of chronic inflammation. They have detected prionuria in scrapie infected mice with coincident chronic lymphocytic nephritis. Transmission has been shown upon intracerebral inoculation of purified proteins from pooled urine collected from scrapie sick or presymptomatic mice. In contrast, prionuria was not observed in scrapie infected mice displaying isolated glomerulonephritis without interstitial lymphofollicular foci or in scrapie infected wild type mice lacking inflammatory conditions.

Prionuria was also detected in chronic wasting disease (CWD) of deer. Experiments by Haley *et al.*[72] provided evidence that concentrated urine from deer at the terminal stage of the disease, that also showed mild to moderate nephritis histopathologically, was infectious when inoculated into transgenic mice expressing the cervid PrP gene. In addition, the urine collected from the CWD sick deer that was used for mouse inoculation, showed positive results when assayed for PrP^{TSE} by serial rounds of protein misfolding cyclic amplification (PMCA) assay. The concentration of abnormal prion protein was very low as indicated by undetectable PrP^{TSE} by traditional assays and prolonged incubation periods and incomplete TSE attack rates in the transgenic mice.

Using the highly sensitive PMCA technology Gonzalez-Romero *et al.*[73] and Murayama *et al.*[74] have detected PrP^{TSE} in urine of scrapie sick hamsters. The results by Gonzalez-Romero *et al.* suggest that the concentration of PrP^{TSE} in urine is in average 10-fold lower than in blood. Animal experiments have demonstrated that *in vitro* generated PrP^{TSE} by PMCA starting from urine produced a disease indistinguishable from the one induced by infected brain material.[73]

Epidemiological evidence in the last 25 years, during which urinary-derived medicinal products and particularly gonadotrophins have been widely used, does not suggest a risk from sporadic CJD. Since epidemiological evidence has identified the few cases of iatrogenic transmission of CJD through the use of pituitary-derived gonadotrophins, it is possible that transmission from urinary-derived gonadotrophins would have been detected if it had occurred.

9. Recommendations and proposals

9.1. Sporadic, genetic and iatrogenic CJD and plasma-derived medicinal products

Cumulative epidemiological evidence does not support transmission of sporadic, genetic and iatrogenic CJD by blood, blood components or plasma-derived medicinal products.[75, 76, 12] Nevertheless, rigorous epidemiological studies for tracing blood-related sCJD cases have not yet reached sufficient statistical power to formally exclude the possibility of blood transmission in a small number of cases. Moreover, the experimental evidence of peripheral tissue infectivity in various subtypes of sCJD is very limited but available data show presence of infectivity in spleen and lymph nodes in human TSEs other than vCJD.

The implementation of appropriate actions in relation to CJD depends on accurate diagnosis in suspected cases. There is a potential for diagnostic confusion between sporadic and variant CJD, particularly in younger age groups.

Donor selection criteria include criteria to exclude donors who might be at higher risk of developing CJD. The following permanent deferral criteria are specified in Commission Directive 2004/33/EC: Persons who have a family history which places them at risk of developing a TSE, or persons who have received a corneal or dura mater graft, or who have been treated in the past with medicines made from human pituitary glands.[7b] Precautionary recalls of batches of plasma-derived medicinal products after post-donation reports of CJD or CJD risk factors in a donor contributed to severe shortages of certain products.[9a]

On the basis of the current epidemiological evidence, the CHMP recommendation that recall of plasma-derived medicinal products is not justified where a donor is later confirmed as having sporadic, genetic or iatrogenic CJD or CJD risk factors is maintained.

9.2. Variant CJD and plasma-derived medicinal products

Uncertainties still exist concerning the number of cases of vCJD that will occur although the number of cases is in decline in the UK and France. Variant CJD has a different distribution of infectivity in tissue outside the central nervous system to sporadic CJD.

There is now strong epidemiological evidence of human to human transmission of vCJD by blood transfusion (see Section 4.2). In addition, one vCJD infection was detected in a patient with haemophilia treated with high doses of intermediate purity factor VIII. Estimates of the relative risks of exposure through diet, surgery, endoscopy, blood transfusion and receipt of UK-sourced plasma products suggest that the most likely route of infection in the patient with haemophilia was receipt of UK plasma products. At least one batch came from a pool containing a donation from a donor who later developed vCJD.[43,77]

The following measures are aimed at minimising the risk of transmission of the agent by plasma-derived medicinal products.

9.2.1. Exclusion Criteria

a) Consideration of Country-based exclusions

There is currently no screening test to detect donors who may be incubating the disease or in the early clinical stages. Therefore, other approaches are considered in order to try and identify donors who may present a higher risk.

UK plasma

Residence in the UK is a recognised risk factor for vCJD and has led to the UK deciding no longer to fractionate from UK plasma.

Exclusion of donors based on cumulative period of time spent in the UK

Since UK donors are excluded from donating plasma for the manufacture of plasma-derived medicinal products in the UK, it is consistent to exclude donors who have spent long periods in the UK. This is supported by the finding of vCJD cases, which have a risk factor of long periods spent in the UK, in other countries[5].

It is, therefore, recommended that donors who have spent a cumulative period of 1 year or more in the UK between the beginning of 1980 and the end of 1996 are excluded from donating blood/plasma for fractionation. Countries are highly encouraged to choose their national cumulative period limit for plasma-derived medicinal products according to a nationally calculated benefit/risk balance, which will take into account the endogenous risk of BSE exposure (and introduction in the food chain) and the risk of shortages of blood and plasma for the manufacture of medicinal products. The national limit is recommended to be of cumulative periods in the UK below or equal to 1 year.

Countries may still apply a stricter limit than 1 year for exclusion of donors for blood/plasma collected for fractionation within the country (e.g. 6 months) but will accept plasma-derived medicinal products from other countries provided that at least the one-year time limit is applied.

The rationale for this recommendation is to exclude donors who have the highest individual risk from stays in the UK and to be consistent with the UK decision to no longer fractionate from UK plasma. This is further explained in the first version of this Position Statement published in February 2003.[5b]

French plasma and plasma from other BSE-exposed European countries

France published an analysis of the risk of transmission of vCJD by blood and its derivatives sourced from French plasma in December 2000.[78g] This concluded that plasma collected in France could continue to be used for fractionation. The safety margin for plasma-derived medicinal products was considered to be sufficient. However, introduction of additional steps to further increase the safety margin of some products was recommended (e.g. nanofiltration of Factor VIII introduced in January 2001). Leucodepletion for plasma for fractionation, as for plasma for transfusion products, was also recommended in 2001 as a precautionary measure. The subsequent risk-analyses published in 2002, 2003, 2004, 2005, 2007 and 2009 re-confirmed these conclusions and acknowledged that the size of epidemic was revised to a lower estimate by more recent modeling, and the risk to collect blood from vCJD-incubating donors lower than previously estimated.[78]

Based on the limited data on human exposure to BSE-risk materials in other European countries it is still difficult to estimate the epidemiological risk in those countries which have small number of vCJD cases or have not yet reported any vCJD cases.

Donors who have spent a cumulative period of time in France and other BSE-exposed countries

Exclusion of donors who have spent a cumulative period of time in France is not recommended because of the lower risk associated with time spent in France compared with time spent in the UK (the risk in France is estimated to be 1/10 of that in UK). Since the previous version of the Position Statement, endogenous vCJD cases occurred in some other countries (see Section 2. Human TSEs current status) placing them close to or lower than France in terms of incidence and ratio of risk in

[5] Two cases in Ireland, two cases in US, one case in France and the Canadian case associated with long periods spent in the UK.

comparison to UK. Exclusion of donors who have spent time in other European countries having a risk ratio in the same order of magnitude as France is not recommended.

Concluding remarks

Country-based exclusions may appear unjustified in the sense that the vast majority of donors who will be excluded will not develop the disease. There is a lack of spare plasma capacity to make up for shortfalls if countries that are major producers of plasma-derived medicinal products discontinue the use of nationally collected plasma for fractionation.

b) Other possible exclusion criteria

Commission Directive 2004/33/EC indicates that further deferral criteria for vCJD may be recommended as a precautionary measure.[7b]

Other possible exclusion criteria that could be considered include permanent exclusion of recipients of blood transfusion (general exclusion or exclusion of recipients of transfusion in UK[6]), transplant recipients, and donors who have undergone neurosurgery.

Caution is needed because of the risk of loss of donors and consequent supply problems. Since such criteria could apply to both blood and blood components, and plasma-derived medicinal products, it was appropriate to consider this further within the scope of Directive 2002/98/EC.[7a] The technical meeting of blood experts, convened by the European Commission in January 2004, considered exclusion criteria, as well as blood component preparation and processing, recipient tracing and surveillance, and optimal use of blood.[7c]

9.2.2. Leucoreduction and specific prion affinity filters

The benefit of inclusion of leucoreduction to improve the safety of plasma has not been demonstrated.

At present it is not appropriate to recommend the introduction of leucoreduction for the safety of plasma-derived products.

Efficacy of introducing recently developed affinity media / filters is still under investigation.

9.2.3. Manufacturing processes for plasma-derived medicinal products

The available data support the reduction of infectivity by steps in the manufacturing process. Manufacturers are required to estimate the potential of their specific manufacturing processes to reduce infectivity. This should follow a step-wise approach as described below and illustrated in the accompanying flow diagram. It is recommended that manufacturers consult the relevant competent authorities at each of the milestones in this estimation. A decision to undertake an infectivity assay and/or to add a further manufacturing step(s) to increase reduction capacity should only be made after a careful consideration of all benefit-risk factors for a certain product.

Firstly, manufacturers should compare their own processes to those with published data on reduction of infectivity in order to estimate the theoretical potential of their specific manufacturing processes to reduce infectivity. *(Flow diagram, step 1)*

Whereas the general information available on manufacturing processes provides useful background information, the actual effectiveness of a manufacturing process might be dependent on the specific process conditions. Manufacturers should consider the relevance of the published data to their specific manufacturing processes and whether the removal capacity can be expected to be comparable.

If it cannot be concluded that the removal capacity would be expected to be comparable, it is recommended that manufacturers undertake product-specific investigational studies on key steps in their manufacturing processes using biochemical assays. Priority should be given to studies on products with the lowest potential removal capacity. *(Flow diagram, step 2)*

Investigations using biochemical assays may be sufficient if a clear correlation with infectivity data has already been established for similar processes (e.g. ethanol fractionation). If such a correlation is not established (e.g. a novel step) and the step is considered critical for removal of infectivity for the specific product (e.g. it is the only step for removal), the investigations should be confirmed using an infectivity assay for the critical step(s). *(Flow diagram, step 3)*

The above steps will allow manufacturers to estimate the reduction capacity of their manufacturing processes. *(Flow diagram, step 4)*

In cases where the overall reduction capacity is limited, manufacturers should consider the addition of steps that may increase the removal capacity where this is feasible without compromising the safety, quality and availability of the existing products. Discussion with the relevant competent authorities is recommended. *(Flow diagram, step 5)*

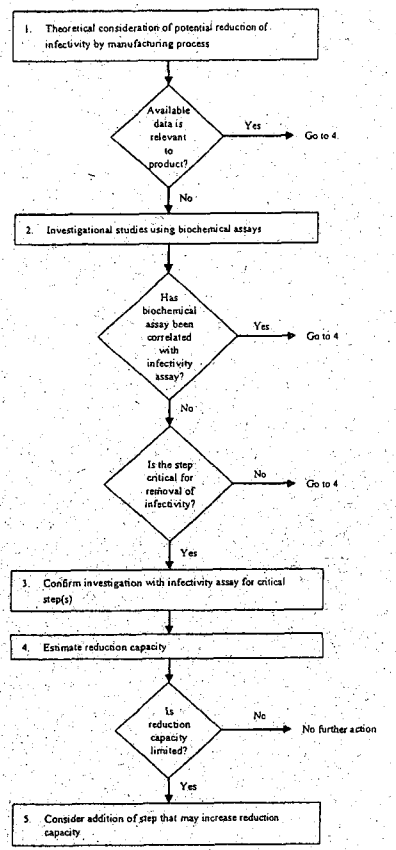
The outcome of the estimates of the theoretical potential of manufacturing processes to reduce infectivity and the results of product-specific investigational studies should be reported to the relevant competent authorities for the medicinal products concerned, as information becomes available. Applicants submitting new marketing authorisation applications for plasma-derived medicinal products will be expected to include such information in the application dossier. The outcome of the estimation of the theoretical potential to reduce infectivity should always be included in the application.

In support of these recommendations, CHMP's Biotechnology Working Party, with the involvement of external experts, has developed guidance on how to investigate manufacturing processes with regard to vCJD risk.[5a]

[6] In April 2004, the UK implemented exclusion of persons who have previously received transfusions of whole blood components since January 1980, as a precautionary approach.

Figure 1: Plasma-Derived Medicinal Products: estimation of potential reduction capacity of specific manufacturing processes

Important Note: this flow diagram should be read in conjunction with the preceding text in 9.2.3. It is recommended to consult the relevant competent authorities at the milestones in this estimation. Give priority to studies on products with the lowest potential removal capacity.



9.2.4. Recall of batches where information becomes available post-donation

In view of the lack of adequate information on vCJD, it is prudent to recall batches of plasma-derived medicinal products where a donor to a plasma pool subsequently develops vCJD. Recall should also include medicinal products containing plasma-derived products as excipients. However, in both cases, consequences for essential medicinal products where alternatives are not available will need careful consideration by the competent authorities.

A case-by-case consideration would be appropriate where plasma-derived products have been used in the manufacture of other medicinal products. This consideration would include the nature of the product, the amount used, where it is used in the manufacturing process and the downstream processing.

Look-back to identify the fate of donations should be taken as far as possible. Regulatory authorities, Official Medicines Control Laboratories, surveillance centres and the supply chain should be informed of all batches of product and intermediate implicated whether or not supplies of the batch are exhausted.

There is no recommendation to recall batches if information becomes available post-donation, which would have excluded a donor based on his/her stay in the UK since this donation exclusion criteria is a very conservative precautionary measure (see 9.2.1).

9.2.5. Albumin used as an excipient or in manufacturing processes

The available data on the removal of infectivity during the fractionation process used in the manufacture of albumin indicates that the risk of transmission of infectivity by albumin would be particularly low. Nevertheless, in the case of albumin used as an excipient, recall is still recommended as a precautionary measure where a donor to a plasma pool subsequently develops vCJD. A single batch of albumin may be used to produce a number of batches of a medicinal product because of the small amounts that are typically used as an excipient. As a consequence, a recall could affect complete stocks of a product and create severe shortages. Therefore, to avoid a negative impact on supply, companies should consider the origin of plasma and select countries where the probability of having to recall batches is as limited as possible.

Development of substitutes for plasma-derived albumin used as an excipient or in manufacturing processes is encouraged although it is recognised that this can be difficult (requiring development and validation and usually non-clinical and clinical investigations) and should thus be considered as a long-term approach.

9.2.6. Substitution with alternative products

Use of alternative products to plasma-derived medicinal products could be considered, where these are available. It is felt that this choice should remain with users, taking into account the needs of the individual patient. It should be noted that plasma-derived products such as albumin may be used in the manufacture of recombinant products.

9.2.7. Optimal Use

Optimal use of plasma-derived medicinal products is encouraged, as this will maximise the benefits of the products compared with any potential risk.

9.3. Urine-derived medicinal products

The recommendations for urine-derived medicinal products are based on the following considerations:

There is no epidemiological evidence of CJD and vCJD transmission by urine-derived medicinal products.

TSE infectivity in urine has been reported in some animal models.

The review of manufacturing processes described below.

Investigational studies of infectivity reduction by the manufacturing processes should be done following the same general, stepwise approach as recommended for plasma derived medicinal products (see Section 9.2.3).[5a]

Results from different assay systems are not necessarily directly comparable (Western blot, cell based assays, bioassay). The approach recommended for plasma-derived medicinal products would be applicable (i.e. confirm reduction capacity using infectivity assays for steps critical for reduction of infectivity if a clear correlation between data from biochemical assays and infectivity assays has not been established for similar process steps). For inactivation studies, investigation of different TSE strains should be considered as they may vary in resistance.

Potential accumulation of prions on chromatographic columns or a potential batch to batch *contamination due to carry-over of prions should be addressed in the studies.*

Bibliographic data could be acceptable as additional supportive data to the investigational studies provided. Similarity of the compared process and materials should be established. Extrapolation of results for plasma-derived medicinal products is not justified particularly for chromatographic steps at the beginning of the manufacturing process because of the high protein content in plasma.

General review of the manufacturing processes indicates that, in each manufacturing process, there is at least one step that might be theoretically capable of reducing infectivity if it were present in the starting material. In cases where the reduction capacity is limited, manufacturers should consider the addition of steps that may increase the overall removal capacity.

For particular products, such as hormones from a relatively small well-defined donor population, some manufacturers have put in place limited exclusion criteria for the selection of a donor for inclusion in a *donor panel. For other products manufactured from very large donor pools (e.g. urokinase), such* measures are more difficult to apply.

Urine should be collected from countries where there is a surveillance system for both human and animal TSEs. It is noted that urine-derived medicinal products are not sourced from urine collected in the UK.

On the basis of these considerations, the use of exclusion criteria for selection for a donor panel are encouraged, as a precautionary measure, where feasible. The same exclusion criteria should be applied with respect to CJD and vCJD as used for blood/plasma donors providing starting material for the manufacture of plasma-derived medicinal products. Although these criteria would not be checked at each donation unlike blood/plasma donors, manufacturers should follow up the donor criteria at defined intervals. The exclusion of donors with known inflammation of kidney and/or chronic renal inflammatory diseases is encouraged.

Record keeping for traceability is recommended for products where it is possible to trace back to donor level.

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別紙様式第２－１

医薬品　研究報告 調査報告書

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研究報告の概要

HIV や C 型肝炎をはじめとする血漿製剤によるウイルス感染のリスクは、供血者の選別および検査の導入、ならびに 1986 年の有効なウイルス不活化工程の採用以降、実質的に排除された。
しかし、vCJD の発生後、英国製血液製剤・血漿製剤の安全性に関する新たな懸念が持ち上がり、感染および二次感染拡大のリスクを最小限に抑えるため、2004 年に、後に vCJD を発症したドナーから採取された血漿を含んでいるかどうかにかかわらず、1980 年から 2001 年までの間に英国でプールされた血液凝固因子製剤を投与された患者全員にそのことが通知された。
通知以降、英国における vCJD の新規臨床症例は減少し、過去に関係する血液または血液製剤の投与を受けたことが確認されている vCJD 患者は見つかっていない。
しかし、一般母集団における無症候性 vCJD 感染の有病率は不明であり、適切で有効な vCJD のスクリーニング試験がいつ頃利用可能になるかは不明である。
血友病患者において最近確認された 1 例目の無症候性の vCJD 感染症例ならびにメチオニン／バリン異型接合患者における vCJD 報告は、遺伝性出血性疾患患者をはじめとする「リスクのある」母集団における継続調査が必要であることを示している。
供血者における vCJD に関連する異常プリオンタンパク質の保有率が不明であること、有効な試験法がないことを考え合わせると、現在実施されている対策を続けることが、出血性疾患患者を含む患者における vCJD 二次感染の拡大を減らす最善の手段であると思われる。

使用上の注意記載状況・その他参考事項等

重要な基本的注意
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。

報告企業の意見	今後の対応
英国における vCJD 対策に関する報告である。 現時点まで血友病以外で血漿分画製剤から vCJD 伝播が疑われた報告はなく、血漿分画製剤の製造工程でプリオンが除去できるとの情報もある。 なお、当社血漿分画製剤の原料血漿は現在まで英国の血漿を使用していない。	今後とも vCJD に関する安全性情報等に留意していく。

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ORIGINAL ARTICLE *Transfusion transmitted disease*

Risk reduction strategies for variant Creutzfeldt–Jakob disease transmission by UK plasma products and their impact on patients with inherited bleeding disorders

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Summary. The appearance and rapid evolution of BSE in UK cattle in the mid 1980s, with compelling data supporting variant Creutzfeldt–Jakob disease (vCJD) as its human manifestation, pose a potentially severe threat to public health. Three clinical cases and one asymptomatic case of vCJD infection have been reported in UK recipients of non-leucodepleted red cell transfusions from donors subsequently diagnosed with vCJD. Plasma from both these and other donors who later developed vCJD has contributed towards plasma pools used to manufacture clotting factor concentrate. The United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) Surveillance Study has detected asymptomatic vCJD postmortem in a haemophilic patient treated with UK plasma products including two batches of clotting factor linked to a donor who subsequently developed vCJD. Over 4000 bleeding disorder patients treated with UK plasma products are recorded on the UKHCDO National Haemophilia Database. The risk of vCJD transmission by plasma products is not known. However, public health precautions have been implemented *since* 2004 *in all* UK inherited bleeding disorder patients who received UK-sourced plasma products between 1980 and 2001 to minimize the possible risk of onward vCJD transmission. We evaluate vCJD surveillance and risk management measures taken for UK inherited bleeding disorder patients, report current data and discuss resultant challenges and future directions.

Keywords: haemophilia, inherited bleeding disorders, UK plasma products, variant Creutzfeldt–Jakob disease

Introduction

The first reports of a prion disease in humans, Creutzfeldt–Jakob disease (CJD), appeared in the 1920s [1,2] with a distinct clinico-pathological variant being described in 1996, variant Creutzfeldt–Jakob disease (vCJD) [3] in which significant involvement of lymphoreticular tissues was demonstrated [4,5]. Compelling epidemiological, clinical, neuropathological and experimental data support vCJD as the human manifestation of bovine spongiform encephalopathy (BSE) [4,6,7], an epidemic of which occurred in UK cattle in the 1980s and early 1990s. The incidence of BSE peaked in 1993, and while the precise origin of the BSE epidemic remains unclear, there is little doubt that the rendering practices employed at that time significantly contributed to its rapid spread throughout the UK. Feeding cattle and sheep ruminant-derived protein was

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banned in 1988 [8], with an ensuing fall in the number of BSE cases. However, such measures were not taken in time to prevent the introduction of BSE-infected cattle carcasses into the human food chain. By January 2010, 167 clinical cases of vCJD attributable to dietary exposure had been reported in the UK by the National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU), a majority of which have been confirmed by neuropathological examination [9]. Much lower but increasing numbers of cases have been reported worldwide, the majority of which are believed to have contracted vCJD in their country of origin [10], probably as a result of the export of UK animals and/or ruminant feed. Although the annual incidence of clinical vCJD in the UK has been steadily declining since 2000 and the extent of the primary vCJD outbreak has been several magnitudes less than previously predicted [11,12], limited information is available to provide accurate estimation of the number of future clinical cases. Where genetic information is available, all confirmed clinical cases of vCJD have thus far been shown to be homozygous for the methionine residue at codon 129 of the prion protein gene (*PRNP*). However, a suspected clinical case of vCJD in an individual heterozygous for methionine/valine has recently been reported [13].

Transfusion transmission of vCJD: early perception of risk, risk reduction measures and plasma product recalls

Distinct from the number of new clinical cases is the unknown prevalence in the UK of presymptomatic, or subclinical, vCJD infection, i.e. where asymptomatic individuals harbour vCJD infection as discussed elsewhere [10,14]. It is from this group of individuals that the risk of secondary vCJD transmission arises, with the characteristic prominent lymphoreticular phase giving rise to the possibility of transmission via surgical instruments, blood and blood products and organ (including bone marrow) transplantation. This differs from classical sporadic CJD, which has been shown to be transmissible by neurosurgical instruments, pituitary derived hormones and corneal transplants but in which transmission by blood or blood products has not been demonstrated [15–21]. The widespread transmission of hepatitis C and human immunodeficiency virus (HIV) infections by plasma products prior to 1986 raised ongoing concerns about the possible emergence of new blood-borne pathogens. These led to the publication of therapeutic guidelines by the United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) recommending, where possible, that plasma-derived factor VIII (FVIII) and factor IX (FIX) concentrates be replaced with recombinant products in the treatment of patients with haemophilia A and B [22]. The first report of clinical vCJD cases in 1996 [3] raised concerns amongst UK haemophilia clinicians that the infective agent may be transmissible by blood products [23]. Around the same time, a collaborative study, the Transfusion Medicine Epidemiology Review (TMER), was established between the NCJDSU and the four UK blood services (UKBS) with the aim of identifying any association between CJD (including variant) and blood transfusion [24]. At that time, 17 patients were recorded as having donated blood prior to being diagnosed with vCJD and there was concern that there may be many more infected, yet asymptomatic individuals amongst the donor population. It was estimated that even a modest prevalence of vCJD in the general population could result in an infected donation entering the plasma pools from which clotting factor concentrates were prepared. Together with the almost exclusive restriction of vCJD to the UK at that time, these concerns greatly influenced the UKHCDO's decision in 1997 to recommend the use of bovine material-free recombinant products, as well as fractionated products from non-UK plasma donations [23]. Treatment with recombinant factor concentrates was funded in 1998 for haemophilic patients aged <16 years and was extended to include all adult patients by ascending age from 2003/2004 and completed in 2005/2006.

In the absence of a test to detect preclinical vCJD infection, a number of precautionary donor selection and component processing measures have been introduced since 1998 to minimize the possible risk of secondary vCJD transmission by blood and its components (Table 1) [25–29]. The uncertainty of vCJD transmissibility by plasma products led to the recommendation by the Committee for Proprietary Medicinal Products that a product be recalled where a donor subsequently diagnosed with vCJD had contributed to the plasma pool (termed an 'implicated' batch) [30]. In 1997, there were two Bio Products Laboratory (BPL, the plasma fractionator for the UK National Blood Service) recalls of clotting factor concentrates [31], both of which included batches of in-date FVIII concentrate.

The first risk assessment of plasma vCJD infectivity

Theoretically, the degree of exposure of an individual recipient to vCJD infection is dependent on the prevalence of subclinical infection within the donor

Table 1. Measures taken in the UK to minimize risk of variant Creutzfeldt–Jakob disease (vCJD) transmission by blood and plasma product transfusion.

I. Rationalization of clinical use of blood and blood products. Department of Health initiatives: Better Blood Transfusion 1998, 2002, 2007
II. Donor selection
a. Use of non-UK donors for plasma product fractionation (announced 1998, implemented 1999)
b. Use of non-UK plasma donors in under 16 s or adult recipients of large plasma volumes (2002)
c. Exclusion of recipients of blood transfusion since 1980 from donor pool (2004)
d. Exclusion of individuals from donor pool who are unsure whether they have received a blood transfusion since 1980 (2004).
e. Exclusion of donors where recipients have developed vCJD where blood transfusion cannot be excluded as source of vCJD and where no infected donor has been identified (2005)
III. Component processing
a. Leucodepletion of all blood products to white cell concentration $<10^6$ L^{-1} (announced 1998, implemented 1999)
b. Use of recombinant factors in selected patients with haemophilia A and B (1998) and all others (2003–2005)
IV. Product recall where donor confirmed as suffering from vCJD found to have contributed to plasma pool

Table 2. Possible determinants of risk of variant Creutzfeldt–Jakob disease (vCJD) transmission by transfusion of blood and plasma products.

I. Levels of infectivity in donor population
a. Prevalence of sub-clinical infection – geographical variation
II. Exposure of recipient to infected donors
a. Infectivity of donation within incubation period
b. Quantity of plasma/leucocytes within component
c. Number of donors contributing towards component/size of plasma pool
d. Number of transfusions received
e. Manufacturing process: e.g. leucodepletion, plasma fractionation, inactivation procedures
III. Susceptibility of recipient
a. Genotype e.g. codon 129 *PRNP*
b. Age
c. Other

population, the manufacturing process of a given blood component and the number of transfusions received (Table 2). The partitioning of prion infectivity during the manufacture of plasma products has been extensively investigated and is reported elsewhere [32–37]. In addition, there is individual variation in susceptibility to infection, with possible influences including age and *PRNP* genotype. An independent assessment of the risk to patients of exposure to vCJD infectivity in blood products was carried out on behalf of the Department of Health (DH) by Det Norske Veritas Consulting (DNV) and reported in 1999 [38]. To estimate the numbers of new infections and possible resultant vCJD cases, the authors attempted to estimate the proportion of UK blood donations that may be infected with vCJD, the possible level and distribution of vCJD infectivity in blood components and plasma products derived from those donations and the likely level of exposure to infectivity of defined sets of patient groups. Substantive data surrounding several of the variables used in these calculations were lacking, necessitating various assumptions and that data be extrapolated from spiked animal models [39,40]. Based on the assumption that blood is equally infective throughout the incubation period of the disease, the likely proportion of infected donations was estimated as between 1/200 and $1/10^6$, depending on the median incubation period of the disease. Over the same range of infected donations, the recipient's risk of infection was predicted to range between unity and $1/10^6$, depending on the patient group. Each infected donation was estimated to result in 2.6 infected recipients (assuming roughly equal contributions from red cell and plasma product transfusions), approximately 80% of whom may live long enough to develop vCJD [38].

The subsequent confirmation of a further clinical case of vCJD in an individual whose blood donations had previously contributed towards plasma pools resulted in a further BPL recall in 2000 [41]. Unlike the 1997 recalls, all batches of clotting factor concentrate had passed their respective expiry dates at the time of this recall. In Scotland, two donations from an individual later diagnosed with vCJD had contributed to the Scottish National Blood Transfusion Service (SNBTS) fractionation pools, and the affected FVIII and FIX products that had been issued to centres in Scotland and Northern Ireland between 1987 and 1989 were described in the SNBTS notification of 2001.

Management of early plasma product recalls

At the time of the 1997 and 2000 BPL and 2001 SNBTS notifications, the haemophilia centres issued with implicated batches of clotting factor were asked to return any remaining stock and recall any remaining unused batches supplied to patients. No public health precautions were advised at the time of these recalls. The 1997 product recall letters from BPL to haemophilia centres cited the following advice that had been provided by the ethics committee local to the NCJDSU: 'the recipients (patients) should not be informed that the product that they

had received has been recalled for this reason [subsequent diagnosis of vCJD in donor] [31]'. In response to queries raised by clinicians and hospital trusts about this directive, the DH confirmed to medical directors that patients who had received implicated blood products should not be informed [42]. This was based on three considerations: first, that it was not known (the word used was 'unlikely') whether vCJD was transmissible by blood products; secondly, that there was no diagnostic test in existence, and finally that no preventative treatment was available. The consensus given by the DH at the time was that patients would 'not benefit from this knowledge, and that uncertainty created by informing patients could cause unjustified worry and create a permanent blight on their lives' [42]. However, many haemophilia physicians either directly informed patients who had received an implicated batch, or provided all their patients with information about vCJD, giving them the option to be informed whether or not they had received an implicated batch(es). In the case of paediatric patients, parents were similarly contacted. The establishment of the CJD Incidents Panel (CJDIP) in 2000 on behalf of the Chief Medical Officer provided an independent expert committee that advised on issues involving possible vCJD transmission in healthcare settings.

vCJD surveillance in UK patients with inherited bleeding disorders

Over 20 000 UK patients with inherited bleeding disorders are currently registered on the National Haemophilia Database (NHD) of whom around one-fifth have been treated with clotting factor concentrate derived from UK-sourced plasma donations. A pilot retrospective histopathological study of the brains of 22 haemophilic patients who died of HIV-related illnesses during part of the period of potential vCJD infection showed no evidence of vCJD [43]. A 5-year surveillance study of patients with haemophilia was commissioned and funded by the DH in 2000 and co-ordinated by the UKHCDO following ethical approval being given by the London Multi-Centre Research Ethics Committee (MREC/01/2/11). The aims of this study were to determine the extent of exposure of individual patients with inherited bleeding disorders to implicated batches of clotting factor concentrate, to analyse tissue biopsies and autopsy material for vCJD and to notify possible and confirmed clinical cases of vCJD in the UK haemophilic population. It was hoped that all haemophilic patients undergoing surgical procedures involving the central nervous system and lymphoid tissue (including tonsil, lymph nodes and spleen) would consent to participate in the study. It was anticipated that in addition to facilitating the appropriate monitoring and long-term follow-up of patients, the findings from this study would inform future assessments of the risk of vCJD transmission posed by plasma products. The control group comprised haemophilic patients who had not received known implicated batches of clotting factor. At the outset of the study, haemophilia centres were provided with details, including issue dates of known implicated BPL or SNBTS batches they had received and requested to provide recipient data identifiable only by the patient's unique NHD number and date of birth. Participation in this study was voluntary. The data to be collected and recorded in a special file on the NHD was the degree of exposure to UK plasma products between 1980 and 2001, including the dates of first and last exposure to an implicated batch and its quantity.

Second risk assessment and CJDIP recommendations

Concern about the possibility of vCJD transmission by blood and blood products was heightened following the demonstration of blood transmission of BSE in a sheep model [44]. Unlike previous experimental models in which prions were inoculated by the intracerebral route, the sheep in this study had been orally infected with BSE and were therefore more representative of the situation in humans. Furthermore, transmission was shown to occur with blood taken during both the preclinical and clinical stages of infection [45].

A second DNV risk assessment undertaken on behalf of DH was reported in 2003 [46]. This was conducted to inform the management of individuals who had received implicated batches of blood and plasma products. The assessment was based on the various published experimental data in animals to model the potential vCJD infectivity in blood and its various components including plasma products [15,45,47]. The assumptions of this risk assessment were accepted by the Spongiform Encephalopathy Advisory Committee, the Committee on the Microbiological Safety of Blood and Tissue, and by the Committee on Safety of Medicines. CJDIP advised that surviving recipients of implicated red cell concentrates be informed and public health precautions implemented to minimize the risk of secondary vCJD transmission. Together with batch-specific manufacturing data, the risk assessment was used by CJDIP to estimate the potential vCJD infectivity in each batch of implicated plasma product. The likely risk

to treated patients was compared with the 'at-risk' threshold developed by CJDIP to guide the management of other 'at-risk' patient groups [48]. If patients had been exposed to a 'threshold' of 1% or greater potential risk of infection over and above the general risk to the UK population believed to have resulted from dietary exposure to the BSE agent, CJDIP advised that they should be notified and requested to take public health precautions. This 1% additional risk equates to an exposure of 0.02 ID_{50}, which is the equivalent level of infection at which public health precautions are implemented for patients exposed to vCJD via surgical instruments [49]. For each of the major assumptions underlying the risk assessment, the most precautionary option was chosen. The implicated plasma products were divided into three groups based on the assessed risk [50]. Amongst those considered to pose a high risk were FVIII, FIX and antithrombin concentrates, of which as little as one vial of treatment led to an exposure in excess of the defined risk threshold. Products in the medium-risk group included those in which exposure to substantial quantities was required to reach the risk threshold such as immunoglobulins, and the low-risk group comprised products with such low levels of potential infectivity as could effectively be ignored as causing any additional vCJD risk. The low-risk group also included some FVIII products that had been manufactured using implicated albumin as an excipient. Details of the majority of batches of implicated plasma products and their distribution directly to centres or through consignees were provided by BPL and SNBTS. To reduce the possibility of onward transmission of vCJD, it was recommended by CJDIP in 2004 that public health precautions be taken in recipients of 'high risk', and 'medium risk' implicated plasma products who had exceeded the 1% additional risk threshold.

Transmission of vCJD by blood transfusion

The CJDIP recommendations to implement public health measures in 'at-risk' recipients of implicated red cell and plasma products were reinforced by the subsequent recognition of the first case of vCJD transmission by blood transfusion [51]. TMER surveillance of the 66 recipients of red cell transfusions derived from the 17 vCJD patients who had previously donated blood has established that of the 24 identified recipients who survived more than 5 years following transfusion, three to date have shown evidence of vCJD infection [52]. In addition to these three clinical secondary cases of vCJD [51,53,54], a further asymptomatic case has been reported, in which the patient died from unrelated pathology with no evidence of neurological disease, but with post-mortem evidence of prion accumulation in lymphoreticular tissue [55]. All affected red cell donations are known to have been made relatively close to the onset of clinical symptoms in the donor, consistent with the increasing level of prion infectivity demonstrated throughout the incubation period in some animal models [56]. The incubation period in these secondary transfusion transmitted cases was around half the length of that estimated for primary oral infections from BSE. All three clinical cases were methionine homozygotes at codon 129 [51,53,54], while the asymptomatic case was methionine/valine heterozygous [55]. As a significant proportion of patients in the TMER recipient cohort did not survive long enough to develop clinical disease should they have been infected by vCJD, it is possible that the observed number of infected recipients underestimates the transmissibility of vCJD by blood transfusion. Likewise, it is possible that other surviving recipients are currently harbouring subclinical infection.

2004 vCJD plasma product patient notification exercise

UKHCDO advice

By the time of the 2004 CJDIP recommendations, the fate of products manufactured from 23 plasma donations derived from nine UK plasma donors who later developed vCJD had been established. These donations had undergone fractionation to produce albumin, immunoglobulin and clotting factor concentrates, including 16 batches of FVIII and eight batches of FIX that were distributed in the UK. TMER surveillance identified that these donations included plasma from at least one donor who, it is likely, had already transmitted vCJD via red cell concentrates [57]. At this time, it was considered likely that further batches of UK-sourced plasma products would become implicated as future vCJD cases arose. Therefore, to prevent secondary spread to other patients a 'population' or 'umbrella' approach was implemented in patients with inherited bleeding disorders who had received UK plasma-sourced products between 1980 and 2001. This policy was advised by UKHCDO and endorsed by CJDIP, DH and the Haemophilia Society, the UK charity representing patients with inherited bleeding disorders. As a result, all patients with bleeding disorders who had been treated with UK-sourced pooled factor concentrates between 1980 and 2001 were considered to be 'at-risk' of vCJD for public

health purposes and precautions were required to minimize the potential risk of secondary transmission. The start date of 1980 was when BSE was believed to have entered the human food chain and the end date of 2001 was the last possible expiry date of any product manufactured by UK fractionators and sourced from UK donors. This approach was based on the assumption that many further vCJD implicated batches of clotting factor concentrate would subsequently be identified and that only small volumes of implicated FVIII or FIX treatment were required for the recipient to be deemed 'at-risk' of vCJD. It was anticipated at that time that extending the 'at-risk' group of patients with inherited bleeding disorders and anti-thrombin deficiency in this way would significantly reduce the risk of secondary vCJD transmission. Such an approach differed from that taken in patients with primary immunodeficiency disorders in whom immunoglobulin forms the mainstay of treatment. As much larger quantities of this product are required to reach the 'at-risk' threshold, individual risk assessments were undertaken in these patients.

National advice: HPA responsibilities

The patient notification exercise was conducted in September 2004 and coordinated on behalf of the DH by the Health Protection Agency (HPA) in England, Wales and Northern Ireland, and the Scottish Centre for Infection and Environmental Health. Several professional and patient organizations, support groups and other stakeholders were involved in the consultation, planning and training for the notification exercise for patients with bleeding disorders, including representatives of UKHCDO, UKBS, the plasma fractionators and the Haemophilia Society. All clinicians responsible for the care of patients with bleeding disorders were provided with information to enable them to notify their patients and advise those for whom public health precautions were required. A date for contacting patients and their general practitioners was specified, which coincided with a national press release. At the same time, the Haemophilia Society informed its members by post about the notification process and provided a fact sheet on vCJD.

Haemophilia clinicians were provided with information sheets and a template letter to patients drafted by HPA/UKHCDO. Haemophilia centres were required to trace all recipients of clotting factors sourced from UK plasma between 1980 and 2001 and document their 'at-risk' status in the patient's medical records including details of exposure to implicated batches. Where a patient's care had been transferred to another centre, clinicians were instructed to forward recipient treatment details to the current centre, which was then responsible for informing the patient. All patients with bleeding disorders were to be notified, provided with written information and given an opportunity to discuss and find out whether they had received UK sourced plasma clotting factors in the specified time period (and were therefore considered 'at-risk'), as well as being given an option to find out whether or not they had received implicated batches. 'At-risk' patients were advised to inform providers of medical, surgical or dental treatment so that appropriate measures could be taken to minimize the risk of secondary vCJD transmission by instruments. They were also advised to inform their families in the event that a future emergency situation should arise and advised not to donate blood, tissues or organs which, in any event, this patient population is precluded from. 'At-risk' patients were advised that their clinical care should not be compromised in any way and invited to discuss the implications of the notification exercise. The original ethical approval was amended to facilitate recording of these relevant data for surveillance purposes on the NHD as previously described. Patients were requested to contact their clinician should they not wish their details to be recorded in this way.

BPL responsibilities

Haemophilia clinicians were contacted directly by BPL or SNBTS with details of any vCJD implicated batches they had been issued. While this accounted for the majority of the implicated batches, the data were incomplete at the time of the 2004 notification exercise, and the eventual tracing of product distribution of FVIII and FIX concentrate issued in 1988 through consignees resulted in a further patient notification in 2006 by which time, this information had become available.

Haemophilia clinician action

All 104 UK haemophilia centres received details of the 2004 exercise electronically 2 weeks prior to the date specified for notifying patients. The notification process comprised the identification of 'at-risk' patients, patient and general practitioner notification, NHD notification, responding to patient reply slips, implementation of patient counselling services and devising hospital procedures through which the public health measures would be implemented. As

'at-risk' patients were identified, any who had recently undergone a surgical procedure involving specified tissues where the instruments used had not yet undergone 10 subsequent cycles of use/decontamination would need to be identified so that advice could be sought from CJDIP regarding the quarantine and handling of these instruments. Pertinent to the notification process was the adoption by hospital trusts of a multidisciplinary approach with collaborative links formed between haemophilia clinicians, infection control services, surgeons, gastroenterologists and others. Education of health care professionals in each hospital trust was imperative to enable the effective implementation of public health policies in 'at risk' patients. The number of patients with bleeding disorders registered at a given centre ranges from single figures to over 1500 and there was significant variation between centres in the resources available to implement the guidance within the specified time period. While the use of electronic records in many centres greatly facilitated the tracing of clotting factor concentrate, these frequently did not cover the early part of the 1980–2001 period, a difficulty that was compounded in some centres by incomplete or unavailable manual records. Infection control policies were informed by guidance from the Advisory Committee on Dangerous Pathogens TSE Working Group [58] and hospital trusts were required to devise means to implement the public health measures in 'at-risk' patients.

Variation in implementation of HPA guidance

Based on local knowledge of their patient group, some clinicians opted to contact only 'at-risk' patients to minimize any possible confusion and prevent unnecessary anxiety in the not insignificant proportion of patients registered with bleeding disorders who had never received UK plasma derived clotting factor concentrate. The UKHCDO requested that haemophilia centres pass on information in situations where patients had moved to another centre. The effectiveness of this varied; some patients were notified by more than one centre, and other patients may have remained untraced as they moved between centres. This difficulty in tracing and contacting patients is now being resolved as the UKHCDO moves towards a data-sharing approach between centres carrying out public health notifications. While there has been no formal evaluation of this notification, there have been anecdotal reports of clinicians notifying only patients known to have received implicated batches of their vCJD risk status. Furthermore, as the notification process requested patients to clarify their 'at-risk' status, it is possible that some patients remain unaware that they pose a public health risk unless specific action has been taken by clinicians to inform them.

A lack of understanding of the nature of the notification process has resulted in some 'at-risk' patients feeling stigmatized, and there have also been instances of patients being incorrectly labelled as having, rather than being at risk of, vCJD. Despite such difficulties, the telephone helplines set up for patients during the notification exercise as well as NHS Direct received few calls. Moreover, the findings from a study of other at-risk vCJD individuals are reassuring; no adverse long-term behavioural or emotional sequelae have been reported in individuals who have either undergone surgery involving contaminated instruments or who have donated blood to patients subsequently diagnosed with vCJD [59].

Endoscopy

A significant challenge that has arisen from the public health notification exercises surrounds endoscopic biopsy. The possible contamination of the biopsy forceps and the endoscope channel as a result of vCJD infectivity in the gut mucosa of subclinically infected individuals [60] led to the 2003 recommendation to quarantine endoscopes and retain their use only for the specified patient should invasive procedures such as biopsy or diathermy be required in an 'at-risk' patient [58]. For several years, the cost implications that resulted from the individualization of endoscopes in 'at-risk' patients requiring biopsy were borne by the hospital trust concerned. This resulted in variation between trusts in the threshold at which biopsies have been performed in these patients, thus raising the possibility that patient care may have been compromised in some cases. In 2008, the DH provided central funding for the refurbishment of suitable quarantined endoscopes used on patients at risk of vCJD [61]. Sufficient resources will similarly be required to ensure the continued implementation of appropriate public health measures in an ageing 'at-risk' bleeding disorder patient population while maintaining high standards of clinical care.

UK products distributed to other countries

As well as being supplied throughout the UK, implicated plasma donations contributed towards pooled plasma products that have been distributed to 13 countries: Belgium, Brazil, Brunei, Egypt, France, India, Ireland, Israel, Jordan, Netherlands, Oman,

Turkey and the United Arab Emirates. It is estimated that patients in at least four of these countries have been exposed to a level of infectivity exceeding the 'at-risk' threshold and the relevant Health Ministries have been contacted by the HPA and informed of the UK approach to risk assessment and patient notification. In the United States, a recent Food and Drug Administration risk assessment has concluded that the risk of vCJD infection from FVIII concentrate is very low [62].

Current results of the notification exercise and UKHCDO surveillance study

Patient exposure to UK plasma products including vCJD implicated batches

The collection of data of patients who received implicated batches and its entry on the NHD remains ongoing and has been greatly assisted by online registration. Annual returns historically provided by haemophilia centres to the NHD detail patient's treatment including product type and adverse events. From these data, it has been possible to estimate the number of patients treated with UK plasma products between 1980 and 2001. Furthermore, details of patient exposure entered into the database have been cross-checked against batch information provided to individual centres by BPL to establish the extent to which implicated batches are accounted for. Recently, similar total data for implicated batches supplied by SNBTS has been provided. This audit indicates that not all of the recipients for some of the batches have been notified to the NHD.

Using the NHD annual data, the estimated number of patients who received UK plasma products between 1980 and 2001 is 4581, of whom 792 are notified as having been treated with one or more than one implicated batch. The units of treatment received by the latter group of patients account for only 12.7 of the 23.7 million units of implicated batches released and 792 is therefore an underestimate of the number of patients treated with an implicated batch. The quantities of each released implicated batch supplied to UK haemophilia centres together with the units accounted for in the notification exercise is presented in Table 3. The percentage of each batch that is accounted for is also shown. For some of these batches, the accounting of use by the patient notification exercise is disappointingly low. The reasons for the low notification of some implicated batches are not known, although patient refusal for the inclusion of their data may be a contributory factor. The last year an implicated batch was identified was 1999 and no further blood donors who donated plasma prior to developing vCJD have been identified since the 2004 notification.

Table 3. Implicated batches of clotting factor concentrate by batch number, product name, release and expiry dates, and units released and used.

Batch number	Product name*	Factor type	Release date	Expiry date	Units released	Sum of units used	% Units accounted for
FHB4116	8Y	VIII	June 1992	April 1995	775 000	280 710	36
FHB4189	8Y	VIII	April 1993	March 1996	1 233 500	735 725	59
FHB4419	8Y	VIII	July 1995	June 1998	1 022 000	656 600	64
FHB4547	8Y	VIII	September 1996	September 1997	902 000	873 821	94
FHB4596	8Y	VIII	May 1997	March 2000	1 398 500	1 054 410	75
FHC0059	8Y	VIII	September 1988	July 1989	528 720	58 560	11
FHC0289	8Y	VIII	May 1990	March 1993	633 500	266 960	42
FHC0369	8Y	VIII	December 1990	October 1993	604 500	199 060	32
FHC4237	8Y	VIII	March 1994	October 1996	1 268 500	982 977	77.4
FJA0020	9A	IX	October 1988	August 1989	533 500	88 025	16
FJA0092	9A	IX	May 1990	April 1991	511 800	92 990	18
FJA4239	9A	IX	July 1993	December 1996	251 000	141 435	56
FJA4308	9A	IX	June 1994	April 1997	573 000	379 540	66
FHM399	High Purity F8	VIII	November 1991	April 1994	812 000	169 055	20
FHM405	High Purity F8	VIII	May 1992	October 1994	905 500	304 500	33
3502–70210	HT DEFIX	IX	Not known	Not known	230 184	216 220	93.9
FHE4437	REPLENATE	VIII	September 1995	July 1997	1 547 000	818 095	52
FHE4536	REPLENATE	VIII	September 1996	July 1998	2 069 000	1 224 270	59
FHE4548	REPLENATE	VIII	October 1996	September 1998	1 690 000	965 400	57
FHF4625	REPLENATE	VIII	July 1997	June 1999	2 290 000	1 035 900	45
FJM4327	REPLENINE	IX	October 1994	February 1996	1 607 500	1 139 915	70
FJM4437	REPLENINE	IX	November 1995	March 1997	832 500	379 380	45
FJM4596	REPLENINE	IX	April 1997	September 1998	838 500	592 380	70
FJM4625	REPLENINE	IX	July 1997	November 1998	875 000	22 145	2.5
0304–70510	Z8	VIII	Not known	Not known	123 690	16 150	13
0301–70320	Z8	VIII	Not known	Not known	125 440	Not known	0

*For further details [see ref. 22].

Tissue-based vCJD surveillance

Following the 2004 notification exercise, the vCJD surveillance study was extended and remains ongoing, although the number referred for postmortem remains low. There were 669 deaths in bleeding disorder patients between 2004 and 2008 including 269 treated with UK plasma products and 37 recipients of implicated batches. However, only a small number of study postmortems have been performed [63]. The report of the first asymptomatic case of probable transmission of vCJD by clotting factor concentrates [63] emphasizes the need for higher recruitment to this study if we are to improve our understanding of the risk of vCJD transmission via infected plasma products. Active vCJD surveillance of prospective tissue samples and autopsy material continues. The Office of National Statistics has provided information about deaths of haemophilic patients including whether the death certificate indicates that a postmortem was or may have been done. This is currently under investigation in the hope of providing further postmortem material for study.

Information to patients (February 2009)

The postmortem arm of the surveillance study has detected PrP^{res} in the spleen of a patient with haemophilia who had had no evidence of any neurological disorder while alive [63]. This patient was known to have been treated with at least one implicated batch of BPL FVIII 8Y. A decision was made to inform bleeding disorder patients of this finding even though the investigation of this case was continuing. A toolkit of letters and information sheets prepared by HPA/UKHCDO was electronically mailed to all Haemophilia Centres with instructions for patients to be informed as soon as possible by post. Many centres decided to post letters to only the patients in the at-risk group.

Further investigation of this patient's complete clinical records showed that he had received treatment with UK-sourced FVIII concentrates including two implicated batches of 8Y, each of which contained a plasma donation from the same donor who subsequently went on to develop vCJD. The patient had also been transfused with 14 units of red cells between 1998 and 2007 and had had invasive endoscopies. Further information about this is contained in a separate paper [63]. Of these potential risk factors, the only link to contact with a patient with vCJD was the two implicated 8Y batches. A further risk assessment by the Department of Health interprets the most likely source of vCJD in this patient as being treatment with UK plasma products [64].

Whilst to date no haemophilia or bleeding disorder patient has been diagnosed with, or died from, clinical vCJD, this information has increased anxiety among some at-risk patients as this is the first information linking treatment with an implicated batch and the detection of PrP^{res} in lymphoid tissue in a patient with haemophilia. However, it is too early to estimate the full implications of these findings on this group and other people treated with blood and plasma products produced in the UK from UK-sourced plasma.

Conclusions

The risk of transmission of viruses by plasma products including HIV and hepatitis C has been virtually eliminated since the introduction of improved donor selection and testing and the employment of effective viral inactivation processes in 1986. However, new concerns regarding the safety of UK blood and plasma products have arisen following the emergence of vCJD. An early precautionary approach was adopted in UK bleeding disorder patients with the aim of minimizing the possible risk of vCJD transmission and its secondary spread. These include their exclusion as blood and tissue donors; an approach that has subsequently been extended to include all recipients of cellular blood products in the UK. Public health measures were implemented in 2004 in all patients who had received UK pooled plasma clotting factor concentrates between 1980 and 2001, irrespective of whether these had contained plasma from a donor known to have later developed vCJD. Challenges have resulted from this approach and these have been discussed in this paper. Our understanding of the risk of vCJD transmission by plasma products has increased over time and informed risk reduction measures. Since the 2004 public health notification exercise, the numbers of new clinical vCJD cases in the UK have declined and no further vCJD patients have been identified as having previously donated implicated blood or blood products. However, the prevalence of subclinical vCJD infection in the general population, including the extent of infection among methionine/valine heterozygotes and valine/valine homozygotes, remains unknown. It is also not known how soon a suitable validated screening test for vCJD

will become available. Although the current risk assessment indicates that only small volumes of implicated clotting factor concentrates are sufficient to cross the additional 1% risk threshold at which public health measures are required, vCJD infectivity amongst implicated batches varies. The recent identification of the first case of asymptomatic vCJD in a haemophilic patient [63] as well as the report of vCJD in a methionine/valine heterozygous individual [13] highlight the need for the continued surveillance of individuals in the 'at-risk' population, including patients with inherited bleeding disorders. Attempts to improve the numbers of postmortem examinations by patients consenting in life or by consent of bereaved relatives needs urgent consideration. Patients who have received implicated batches are currently undernotified to the NHD. Taken together with the unknown prevalence of the abnormal prion protein associated with vCJD among blood donors and the absence of a validated test, continued employment of the population approach appears to be the best means of reducing secondary spread of vCJD between patients, including those with bleeding disorders. Further follow-up may lead to improved understanding of the risk of vCJD to this patient population and the re-evaluation of the current considered 'at-risk' groups for public health purposes.

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医薬品　研究報告 調査報告書

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研究報告の概要

現在までに、後に変異型クロイツフェルト・ヤコブ病(vCJD)を発症した患者からの輸血による vCJD 感染例は４例報告されている。
vCJD を発症した患者からの供血血液を追跡し受血者を特定すると共に、vCJD 登録患者の受血歴と照合したところ、共通の供血者からの輸血が行われていた可能性が示唆された２例について検証した。
患者 A は 2006 年に 18 歳で vCJD により死亡しており、1989 年に新生児特別治療室で４回の輸血を受けていたことが確認されたが、輸血された赤血球(RBC)成分の詳細や供血者を特定できる記録はなかった。
患者 B は 1998 年に vCJD を発症し 41 歳で死亡しており、1993 年６月と 10 月に２度の輸血を受けており合計 103 名の供血者の血液に曝露していた。この 103 名は全て特定され、うち 18 名が患者 A へ輸血された時期にも供血しており、その内１名の供血者の血液が患者 A への輸血が行われた病院に送られていた。
この供血者は、1989 年初頭～2005 年１月まで 26 回の供血を行っており、輸血用に 29 の血液成分(患者 B に輸血された成分および患者 A の入院先へ配給された RBC 成分を含む)と、11 の血漿成分を提供した。血漿成分は 1998 年にイギリス国内血漿の使用が中止になる前に、分画のためバイオプロダクトラボラトリーに供給された。
この２名の患者以外に残りの 27 の血液成分が輸血された患者は特定できなかったが、vCJD 登録患者の中にこの献血者と結びつける受血歴のある患者はいなかった。
一人の供血者からの２例の vCJD 感染の発生を示しているか、または偶然の一致であるかどうかを評価するため、調査を行ったが、２名の受血者の関連性の説明として偶然の一致を除外することはできないが、状況から vCJD を発症しなかった共通の供血者によって結びついた vCJD の血液感染例が新たに２例発生したことが示唆された。

報告企業の意見	今後の対応
vCJD 登録患者のうち共通の供血者からの輸血を受けていた可能性のある２名に関する報告である。 現時点まで血友病以外で血漿分画製剤から vCJD 伝播が疑われた報告はなく、血漿分画製剤の製造工程でプリオンが除去できるとの情報もある。 なお、当社血漿分画製剤の原料血漿は現在まで英国の血漿を使用していない。	今後とも vCJD に関する安全性情報等に留意していく。

使用上の注意記載状況・その他参考事項等
重要な基本的注意 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。

BLOOD COMPONENTS

Variant Creutzfeldt-Jakob disease in a transfusion recipient: coincidence or cause?

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BACKGROUND: To date there have been four instances of infection transmitted through blood transfusions derived from individuals who later developed variant Creutzfeldt-Jakob disease (vCJD). The identification of further transmission of vCJD through this route would have important implications for risk assessment and public health.
STUDY DESIGN AND METHODS: Through the UK Transfusion Medicine Epidemiology Review (TMER) the fate of blood donations from individuals who develop vCJD is traced and recipients of labile components are identified. The details of recipients are cross-checked with the register of vCJD cases held at the National CJD Surveillance Unit (NCJDSU) to identify any linkage between donors and recipients. In the reverse study, when individuals with vCJD are found to have a history of blood transfusion the donors of the transfused blood components are traced and their details cross-checked with the vCJD register to identify any missed or unrecognized linkage between donors and recipients.
CASE REPORT: A case of vCJD has been identified with a history of blood transfusion in infancy. The donors who provided the components transfused cannot be identified, but a blood donor known to have donated blood to another individual who subsequently developed vCJD could have been a donor to the index case.
RESULTS: The at-risk donor is alive 20 years after the relevant donation and continued to donate for some years, until identified as at risk, with 27 other blood components issued for use in patients, none of whom are known to have developed vCJD.
CONCLUSION: Circumstantial evidence has raised the possibility that the case in this report represents a further instance of transfusion transmission of vCJD. However, detailed investigation indicates that the pattern of events may have occurred by chance and disease in this individual may have been caused by transmission of bovine spongiform encephalopathy infection, as is the presumed cause in other primary cases of vCJD.

The Transfusion Medicine Epidemiology Review (TMER) is a collaborative study between the UK National Creutzfeldt-Jakob Disease Surveillance Unit (NCJDSU) and the UK Blood Transfusion Services (UKBS), which was set up in 1997 to identify whether variant Creutzfeldt-Jakob disease (vCJD) was transmissible through blood transfusion. Results from the TMER up to January 3, 2006, have been published.[1] In this article we report on the subsequent identification of a possible link between two vCJD cases who could have received transfusions from a common donor, although this cannot be confirmed.

To date, four instances of probable transmission of vCJD by blood transfusion have been identified by the TMER, including three clinical cases of vCJD and a sub- or preclinical infection.[1,2] Current surviving recipients (n = 21) of blood transfusions derived from individuals

ABBREVIATIONS: TMER = Transfusion Medicine Epidemiology Review; UKBS = UK Blood Transfusion Services; vCJD = variant Creutzfeldt-Jakob disease.

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who later went on to develop vCJD have been informed that they are at greater risk of developing vCJD and, although the level of this risk is uncertain, the four infections through blood transfusion identified to date have developed in a cohort of only 32 individuals who have survived at least 5 years since transfusion.

The identification of further instances of transfusion transmission of vCJD would have important implications for the assessment of risk and for public health. This case report describes the development of vCJD in an individual with a history of blood transfusion derived from donors who cannot be identified but it is possible, based on detailed investigation, that one of the four donors may have been a donor to another vCJD case.

MATERIALS AND METHODS

The TMER study involves the NCJDSU notifying the UKBS of all incident definite or probable cases of vCJD.[1] A search is made via blood donor centers to identify cases that had previously donated blood components and details of the recipients of these components are sought. Identifiers of the recipients are forwarded to the NCJDSU to determine whether any of these individuals appear on the register of vCJD cases. The reverse study involves the NCJDSU notifying the UKBS of all cases of vCJD reported to have received a blood transfusion and the donors are then identified through blood centers and the identifiers checked against the vCJD case register. The study was granted ethical approval by the local research ethics committee.

RESULTS

Case report

Individual A was reported as a case of possible vCJD in 2006 and died of pathologically confirmed vCJD 6 months later, aged 18 years. The age-specific incidence of vCJD in the 15- to 19-year age group is 0.39 per million. The clinical and pathologic features were characteristic of vCJD, which are similar in primary and secondary cases of vCJD. This case was born in 1989 at 29 weeks' gestation and was cared for in a special care baby unit (Hospital X) for approximately 2 months before discharge. The parents reported a history of blood transfusion during the hospital admission, as would be expected in a neonate of this gestation. Staff at the NCJDSU later obtained copies of microfiched medical records, from which it was possible to establish that there were four blood transfusion episodes in early 1989, during the admission to Hospital X. The evidence for these transfusions comprised entries in the clinical notes and records of hemoglobin measurements. However, the medical records contained no details of the red blood cell (RBC) components transfused and no copies of laboratory reports or transfusion records that would allow tracing to specific donors. The hospital blood transfusion laboratory does not have records dating back to 1989.

It has been established that the transfusions were given before the introduction of dedicated RBC units for neonatal use (which allow 1 unit of RBCs to be divided into smaller aliquots and used at different times for the same neonate, thus reducing donor exposure). It is likely therefore that the transfusion episodes represent four different donor exposures. For each of the four transfusions, a small volume of RBCs would have been withdrawn from one adult blood pack.

A second individual (B) developed definite vCJD in 1998 and died at the age of 41 years. The age-specific incidence of vCJD in the 40- to 44-year age group is 0.08 per million. He was identified as having received a total of 103 donor exposures during the course of two separate transfusion episodes between June and October 1993, in a different hospital (Y). Because Hospital X (and thus Individual A) was supplied by the same blood center as Hospital Y and Individual B, the question arose as to whether the two cases might have shared a common donor.

The 103 donors to Individual B have all been identified and assessed as being "at risk of vCJD for public health purposes." Ninety-nine of these donors are alive more than 20 years after the transfusions to Individual A and four have died of causes of death unrelated to CJD or any other neurologic disorder according to their death certificates.

From a review of records of the 103 at-risk donors from 2003, it was established that 18 of the cohort had donated in early 1989, at the relevant time for transfusion to Individual A. The records of these 18 donors were examined to determine whether the RBC components donated in early 1989 were issued to Hospital X, in which Individual A had received the blood transfusions. One such donor has been identified, who donated a unit of RBCs in early 1989, which was issued to Hospital X 6 days later. This unit would have been 13 days old at the time of the first transfusion episode recorded in the medical notes of Individual A or 29 days old at the time of the next transfusion episode.

The pediatrician at Hospital X, who cared for Baby A, has confirmed that in 1989 there were no specific guidelines for top-up transfusions of premature babies. There was no system for allocating a particular unit for sequential top-up transfusions on the same baby, and provision of specific neonatal RBC units only came into place in the mid-1990s. It is probable that, in 1989, standard RBC units would have been provided of blood group O or A, depending on the blood group of the baby. The donor in question is group O. The hospital used "fresher" units by preference, and it is highly unlikely that they would have used 29-day-old RBCs, but the pediatrician could not exclude the possibility that 13-day-old RBCs would have been used for a top-up transfusion. There is therefore a possible common donor to Individuals A and B.

It should be noted that the potential common donor made 26 donations in all, and the early 1989 donation was the first of these. The donor continued donating until January 2005. The 26 donations were processed and provided 29 blood components that were issued for direct clinical use (including the component transfused to Individual B, and the RBC unit issued to Hospital X in February 1989) and 11 plasma components, which were issued to the Bio-Products Laboratory for fractionation, before the use of UK plasma was discontinued in 1998.

To assess whether the potential link observed might be a coincidence rather than reflecting the occurrence of two vCJD transmissions from a single donor, further examination of records was performed to try to establish the likelihood that a randomly selected donor who attended in the relevant time period in 1993 (i.e., the time of the transfusion episodes to Individual B) would, by chance, also have attended in the time period of the transfusions given to Baby A in 1989. Unfortunately, due to a change in record systems at the end of 1992, it was not possible to interrogate individual donor records over these two time periods.

Donor records over similar time periods in later years were therefore examined. For example, we identified donors during the relevant period in 1997 and looked back to determine how many of those donors had donated in the relevant months 4 years earlier (the transfusion to Individual A was in 1989 and Individual B in 1993). This exercise was performed for three combinations of years: 1997/1993, 1998/1994, and 1999/1995.

The results were fairly consistent over the three periods examined, with 10% to 14% of donors donating in both the relevant time periods. Hospital X receives approximately 10% of the blood supply from the blood center in question, so it would be expected that about 10% of the (approx.) 10% who had donated at the right time in 1993 would also have donated at the right time in 1989 with the donation being issued to Hospital X. Thus, the finding that one of the 103 (i.e., roughly 1%) identified donors who donated to Recipient B in 1993 had also donated in the period during which Baby A was transfused in 1989, and whose blood was issued to Hospital X, is just what might be expected by "coincidence," suggesting that coincidence cannot be ruled out as an explanation for the link between the two recipients who developed vCJD.

DISCUSSION

The case of vCJD described in this report (Individual A) received blood transfusions in infancy in 1989, but the donors who provided these components cannot be identified because medical records are incomplete for the period in question. Nevertheless, a blood donor who has been judged to be at risk of developing vCJD, because of a donation transfused to another vCJD case, is known to have donated blood that could have been transfused to Individual A. The question is whether the development of vCJD in Individuals A and B was caused by transmission of infection through blood transfusion from a common, infected donor. While we cannot rule out this possibility, further investigation suggests the observed pattern of events would not be unexpected in the absence of any causal link between the two cases.

Investigation of other donors at the same center indicates that there is a 10% probability that an individual donor would continue to provide blood over a 4-year period and a similar chance that this blood was used in Hospital X where the transfusions to Individual A took place. The fact that one of 103 at-risk donors provided blood on two occasions separated by 4 years and that this blood was used in a particular hospital is therefore not surprising. It is also of note that 48% of blood donors in this region are blood group O, as is the "common" donor and both recipients.

This exercise has highlighted the difficulties in trying to retrospectively link hospital and UK blood service records after an interval of 18 years. The implementation of the Blood Safety and Quality Regulations (2005) enacting a series of EU Directives on quality and safety standards for UK blood establishments and hospitals now means that there is a statutory requirement to ensure that systems are put in place to ensure future full traceability of blood components issued and for these records to be maintained for 30 years. Had this been in place 20 years ago, we would have been able to establish with certainty whether or not Individuals A and B shared a common donor.

The at-risk donor is still alive more than 20 years after the donation potentially transfused to Individual A and this would represent protracted survival in an individual infected with vCJD.[3] In the three clinical cases of established transfusion transmission the two donors developed symptoms of vCJD 17, 21, and 40 months after providing the three implicated donations. However, both these individuals and all three of their infected recipients were methionine homozygous at Codon 129 of the human prion protein gene (*PRNP*), as were Individuals A and B. It is possible that individuals with an alternative genotype at this locus could be infected and survive for many years, and possibly beyond the normal life span, without developing clinical disease.[4] The Codon 129 genotype of the at-risk donor is unknown.

The at-risk donor provided 25 donations between 1989 and 2005, subsequent to the one potentially transfused to Individual A. A total of 28 individual blood components from these 25 donations were issued to hospitals for clinical use, including the transfusion to Individual B. While the fate of the remaining 27 blood components has

not been traced to named recipients, no other cases of vCJD that appear on the NCJDSU database have a history of blood transfusion, which could link them to this donor. Because of the sophisticated CJD surveillance systems that exist in the UK, it is very unlikely that any of the recipients could have developed vCJD but not been reported.

Two of the previous transfusion-transmitted cases received blood from a common donor, with donations separated by 4 months, indicating that it is possible that infectivity in blood in vCJD is sustained through part or all of the incubation period, consistent with some,[5,6] but not all,[7] animal studies. Symptoms of vCJD developed in these transfusion-transmitted cases between 6 and 8 years post-transfusion. Individual A was transfused 17 years before onset of vCJD and the incubation period, if this was transfusion transmission, was therefore more than double that observed previously. However, the transfusions in Case A took place in infancy and there is evidence of reduced susceptibility and extension of incubation periods in neonatal mice experimentally exposed to scrapie as a result of inefficient infection of the immature spleen.[8] If Individual A was infected by blood transfusion, it is surprising that no other recipient from the common donor has developed vCJD, even allowing for some deaths from the underlying condition before symptoms of vCJD might have appeared. Although the Codon 129 genotype of the at-risk donor and the other 27 recipients is not known, approximately 40% of the Caucasian population are methionine homozygous at Codon 129 of *PRNP*.[9]

In conclusion, circumstances raised the possibility that an additional two cases of transfusion-transmitted vCJD have arisen, which are linked by a common donor who has not developed vCJD. The records at the time of the first transfusion are incomplete and an assessment of the likelihood of transfusion transmission depends on a range of considerations, including the chances of blood being provided by a single donor to two recipients in different hospitals, the protracted survival in the donor and Recipient A, and the absence of disease in a cohort of other individuals who received blood transfusions derived from the same donor. Although transfusion transmission cannot be excluded in the case of Individual A, it is also possible that disease in this individual was caused by transmission of bovine spongiform encephalopathy infection through the food chain, the presumed cause of vCJD in other primary cases.[10] The likelihood of food-borne exposure in Case A cannot be estimated directly from the dietary history, but there is evidence of increased susceptibility to primary vCJD in younger age groups.[11]

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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B 個別症例報告概要

○ 総括一覧表
○ 報告リスト

個別症例報告のまとめ方について

個別症例報告が添付されているもののうち、個別症例報告の重複を除いたものを一覧表の後に添付した（国内症例については、資料3において集積報告を行っているため、添付していない）。

感染症定期報告の報告状況(2010/9/1～2010/11/30)

血対課ID	ID	受理日	番号	報告者名	一般名	生物由来成分名	原材料名	原産国	含有区分	文献	症例	医療用
100203	2	2010/9/22	100523	バクスター	乾燥濃縮人血液凝固第Ⅷ因子	乾燥人血液凝固第Ⅷ因子	人血漿	米国	有効成分	有	有	無
100204	3	2010/9/22	100524	バクスター	乾燥濃縮人血液凝固第Ⅸ因子	人血清アルブミン	人血漿	米国	添加物	有	有	無
100215	14	2010/9/29	100548	CSLベーリング	人血清アルブミン 人血液凝固第Ⅹ因子 フィブリノゲン加第Ⅹ因子	人血清アルブミン	ヒト血液	米国、ドイツ、オーストリア	有効成分 添加物	有	有	無
100221	20	2010/10/14	100582	化学及血清療法研究所	乾燥濃縮人アンチトロンビンⅢ	アンチトロンビンⅢ	ヒト血液	日本	有効成分	有	有	無
100238	37	2010/10/27	100667	CSLベーリング	フィブリノゲン加第Ⅹ Ⅲ因子 人血液凝固第Ⅹ Ⅲ因子	人血液凝固第Ⅹ Ⅲ因子	ヒト血液	米国、ドイツ、オーストリア	有効成分	有	有	無
100253	52	2010/10/28	100885	化学及血清療法研究所	フィブリノゲン加第Ⅹ Ⅲ因子 乾燥スルホ化人免疫グロブリン 人血清アルブミン 乾燥濃縮人血液凝固第Ⅷ因子 乾燥濃縮人血液凝固第Ⅸ因子 乾燥濃縮人活性化プロテインC	人血清アルブミン	ヒト血液	日本	有効成分 添加物	有	有	無

別紙様式第4

感染症発生症例一覧

	番号	感染症の種類		発現国	性別	年齢(歳)	発現時期(年/月/日)	転帰	出典	区分	備考			
		器官別大分類	基本語								識別番号	報告日	MedDRA(Ver.)	
第15回	13-2	感染症および寄生虫症	C型肝炎	アメリカ	男性	不明	1999	不明	症例報告	外国製品	09000005	2010/5/27	13.1	第15回症例番号13-2は第13回症例報告番号13-2において報告した
第15回	15-1	臨床検査	HIV検査陽性	アルゼンチン	男性	不明	不明	不明	症例報告	外国製品	03000030	2010/3/30	13.1	
第15回	15-2	感染症および寄生虫症	C型肝炎	ブラジル	男性	不明	不明	死亡	症例報告	外国製品	10000010	2010/6/23	13.1	
第15回	15-2	感染症および寄生虫症	HIV感染	ブラジル	男性	不明	不明	死亡	症例報告	外国製品	10000010	2010/6/23	13.1	
第14回	13-1	感染症および寄生虫症	HIV感染	フランス	男性	49	1983	不明	症例報告	外国製品	08000041	2010/1/19	12.1	第14回症例番号13-1は前回報告における第13回症例番号13-1において報告したものの追加報告
第14回	13-1	感染症および寄生虫症	A型肝炎	フランス	男性	49	1996/5/7	不明	症例報告	外国製品	08000041	2010/1/19	12.1	第14回症例番号13-1は前回報告における第13回症例番号13-1において報告したものの追加報告
第14回	13-1	感染症および寄生虫症	B型肝炎	フランス	男性	49	2003/2/24	不明	症例報告	外国製品	08000041	2010/1/19	12.1	第14回症例番号13-1は前回報告における第13回症例番号13-1において報告したものの追加報告
第14回	13-1	感染症および寄生虫症	C型肝炎	フランス	男性	49	2003/2/24	不明	症例報告	外国製品	08000041	2010/1/19	12.1	第14回症例番号13-1は前回報告における第13回症例番号13-1において報告したものの追加報告
第13回	13-1	感染症および寄生虫症	HIV感染	フランス	男性	49	不明	不明	症例報告	外国製品	08000041	2009/3/18	12.1	
第13回	13-1	感染症および寄生虫症	C型肝炎	フランス	男性	49	1996	不明	症例報告	外国製品	08000041	2009/3/18	12.1	
第13回	13-2	感染症および寄生虫症	C型肝炎	アメリカ	男性	不明	1999	不明	症例報告	外国製品	09000005	2009/5/18	12.1	
第12回	12-1	感染症および寄生虫症	C型肝炎	アメリカ	男性	不明	不明	死亡	症例報告	外国製品	08000023	2008/10/27	11.0	
第12回	12-2	感染症および寄生虫症	急性HIV感染	アメリカ	男性	不明	不明	死亡	症例報告	外国製品	08000023	2008/10/27	11.0	
第11回	5-231	感染症および寄生虫症	HIV感染	ブラジル	男性	不明	不明	死亡	症例報告	外国製品	05000274	2008/4/21	11.0	第11回症例番号5-231は第5回症例番号5-231において報告したものの追加報告
第11回	5-231	臨床検査	C型肝炎ウイルス	ブラジル	男性	不明	不明	不明	症例報告	外国製品	05000274	2008/4/21	11.0	第11回症例番号5-231は第5回症例番号5-231において報告したものの追加報告
第10回	10-1	臨床検査	C型肝炎ウイルス	ブラジル	男性	小児	2004/5/25	不明	症例報告	外国製品	07000015	2007/10/29	10.1	
第10回	10-1	臨床検査	C型肝炎ウイルス	ブラジル	男性	小児	2004/5/25	不明	症例報告	外国製品	07000015	2007/12/28	10.1	追加報告
第10回	10-2	感染症および寄生虫症	急性HIV感染	アメリカ	男性	34	不明	不明	症例報告	外国製品	07000017	2007/12/6	10.1	
第10回	10-2	臨床検査	C型肝炎ウイルス	アメリカ	男性	34	不明	不明	症例報告	外国製品	07000017	2007/12/6	10.1	
第10回	10-3	感染症および寄生虫症	C型肝炎	ベルギー	男性	不明	1991	未回復	症例報告	外国製品	07000028	2008/2/25	10.1	
第9回		0*	0	0	0	0	0	0	0	0	0	0	0	* 当該調査期間に対象となる感染症報告はなかった
第8回	7-012	臨床機査	C型肝炎ウイルス	アルゼンチン	男性	11	2006/5/2	不明	症例報告	外国製品	06000019	2006/9/1	9.0	第8回症例番号7-012は第7回症例番号7-012において報告したものの追加報告
第8回	7-012	臨床検査	C型肝炎ウイルス	アルゼンチン	男性	11	2006/5/2	不明	症例報告	外国製品	06000019	2006/9/25	9.0	第8回症例番号7-012は第7回症例番号7-012において報告したものの追加報告
第8回	7-012	臨床検査	ウイルス負荷増加	アルゼンチン	男性	11	2006/5/2	不明	症例報告	外国製品	06000019	2006/9/25	9.0	第8回症例番号7-012は第7回症例番号7-012において報告したものの追加報告
第7回	7-022	感染症および寄生虫症	A型肝炎	アルゼンチン	男性	不明	不明	不明	症例報告	外国製品	05000648	2006/3/3	8.1	
第7回	7-007	感染症および寄生虫症	A型肝炎	イギリス	男性	不明	不明	不明	症例報告	外国製品	06000013	2006/5/15	9.0	
第7回	7-023	臨床検査	A型肝炎ウイルス	アメリカ	男性	不明	不明	不明	症例報告	外国製品	05000649	2006/3/3	8.1	