資料２－２

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販売名(企業名)	赤血球濃厚液-LR「日赤」(日本赤十字社) 照射赤血球濃厚液-LR「日赤」(日本赤十字社)		Kekre N, Tokessy M, Mallick R, McDiarmid S, Huebsch L, Bredeson C, Allan D, Tay J, Tinmouth A, Sheppard D. Biol Blood Marrow Transplant. 2013 Oct 4. pii: S1083-8791(13)00440-0. doi: 10.1016/j.bbmt.2013.09.013. [Epub ahead of print]	カナダ

研究報告の概要

○全ての血液製剤が白血球除去されている今日、造血幹細胞移植患者への製剤CMV検査は必要か

背景: 造血幹細胞移植(HSCT)患者は、移植後に頻回の輸血を受ける高リスク免疫抑制患者群であり、サイトメガロウイルス(CMV)輸血感染を防ぐために、CMV陰性血液製剤の使用が標準治療とされてきた。近年、血液製剤の白血球除去により、CMV輸血感染リスクが著しく減少することが示されている。カナダでは全製剤で白血球除去が実施されており、CMV検査の必要性が問われている。白血球除去のみ行った製剤と、CMV陰性かつ白血球除去を行った製剤を輸血されたHSCT患者において、輸血感染によるCMV血症の頻度に差があるかを調査した。

方法: 1999年10月1日～2012年6月30日の期間にCMV陰性のドナーから同種HSCTを受けたCMV陰性患者を分析した。CMV血症の定義はPCR陽性とした。

結果: 基準を満たした分析対象166人の患者のうち、89人は白血球除去及びCMV検査で陰性が確認された製剤を輸血されていた2007年1月以前にHSCTを受け、残りの77人はそれ以降にHSCTを受けて、白血球除去のみ行われた製剤を輸血された。これらの2群において、年齢、性別、診断、移植細胞の種類、移植細胞の提供源、移植前処置法、ABO適合性についての差はなかった(P>0.05)。CMV血症は、CMV陰性かつ白血球除去製剤の輸血を受けた患者3人(3.4%)及び白血球除去のみ行った製剤を輸血した患者1人(1.3%, p=0.62)に見られた。CMV血症患者のうち2人がCMV感染症疑い症状を呈したが、いずれもCMV陰性製剤が輸血されていた。総入院期間、集中治療室での治療期間、移植片対宿主病、100日間の非再発死亡率に関して2群間での差はなかった。

結論: 全血液製剤が白血球除去されている今日では、HSCT患者への製剤に対するCMV検査は不要であると考えられる。

報告企業の意見	今後の対応
CMV陰性かつ白血球除去を行った血液製剤の輸血と、白血球除去のみ行った血液製剤の輸血を受けたHSCT患者におけるCMV血症の頻度を比較した結果、2群の間には差は見られず、全血液製剤が白血球除去されている今日では、HSCT患者への製剤に対するCMV検査は不要であると考えられるとの報告である。	日本赤十字社では、CMVの感染防止に有効とされる、保存前白血球除去した血液製剤のみを供給している。さらに、医療機関の要請に応じてCMV抗体(IgG及びIgM)が陰性であることを確認した血液製剤を供給している。今後もCMV感染に関する新たな知見等について情報の収集に努める。

使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-LR「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

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Is Cytomegalovirus Testing of Blood Products Still Needed for Hematopoietic Stem Cell Transplant Recipients in the Era of Universal Leukoreduction?

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A B S T R A C T

Hematopoietic stem cell transplantation (HSCT) recipients are a high-risk, immunocompromised group of patients who receive frequent transfusions after transplantation. Transfusion of cytomegalovirus (CMV)-negative blood products has long been the standard of care to prevent transfusion-transmitted CMV in this patient population. Leukoreduction of blood products before transfusion has been shown to significantly reduce the risk of transfusion-transmitted CMV. In the era of universal leukoreduction in Canada, the need for CMV testing of blood products remains unclear. We sought to identify whether there is a difference in transfusion-transmitted CMV viremia in patients receiving only leukoreduced versus CMV-negative and leukoreduced blood products in HSCT recipients. Patients who were CMV negative and received an allogeneic HSCT from a CMV-negative donor between October 1, 1999 and June 30, 2012 were included in the analysis. Transfusion data were collected from The Ottawa Hospital Blood Bank and Canadian Blood Services. CMV viremia was defined as PCR positivity. One hundred sixty-six patients were identified who met the inclusion criteria. Of these, 89 patients received an HSCT before January 2007, during the time when patients received leukoreduced and CMV-negative blood products. Seventy-seven patients received an HSCT after this time, receiving only leukoreduced blood products. The 2 groups did not differ in terms of age, gender, diagnosis, graft type, graft source, conditioning regimen, or ABO compatibility ($P > .05$). CMV viremia was detected in 3 patients who received CMV-negative leukoreduced blood products (3.37%) and in 1 patient who received only leukoreduced blood products (1.30%, $P = .6244$). Of the patients who developed CMV viremia, 2 developed suspected CMV disease. Both of these patients were transfused with CMV-negative blood products. Secondary outcomes, including total length of stay in hospital, admission to the intensive care unit, acute and chronic graft versus host disease, and 100-day nonrelapse mortality, did not differ between the groups. In the era of universal leukoreduction of blood products, this study demonstrates that testing for CMV-negative blood products is not needed for HSCT recipients.

INTRODUCTION

Transfusion practices over the years have drastically changed to improve the quality and safety of products transfused. One such change has been the implementation of universal leukocyte reduction of all transfused red cell and platelet products in Canada beginning in 1999. This was implemented after leukoreduction was shown in studies to decrease the incidence of febrile nonhemolytic transfusion reactions by approximately half, from .33% to .37% in red blood cell (RBC) [1-3] transfusion and from .18% to .19% in platelet [1,4,5] transfusions. Furthermore, multiple studies have shown that leukocyte reduction decreases the rate of alloimmune platelet refractoriness [6].

Cytomegalovirus (CMV) transmission is a recognized complication of blood transfusions. CMV is a DNA virus that, after primary infection, remains in a latent form. Sites of latency are believed to include bone marrow progenitor cells and monocytes. Prevalence of CMV antibodies in the general adult population ranges from 40% to 100% [7], with some studies showing a higher prevalence among men, people from a lower socioeconomic status, and at an increasing age [8]. In Canada, the seroprevalence has been estimated at 60% to 70% [8]. One Canadian study showed that CMV seroprevalence among daycare workers was associated with increasing patient age, interaction with children, and low income birth country [9].

Transfusion-transmitted CMV infection in transfusion recipients who are immunocompetent is uncommon, reported to be about 1% [10]. This incidence is thought to be higher in immunocompromised patients, including hematopoietic stem cell transplantation (HSCT) recipients [11]. The most significant disease manifestations of CMV in HSCT recipients are pneumonia and gastrointestinal disease, both of which are associated with significant morbidity and mortality in these patients [9]. CMV pneumonia in HSCT recipients has been associated with mortality in 85% to 90% of these patients [12,13]. With pre-emptive treatment with antivirals, this has become much less common [14,15].

Efforts have been made to decrease the risk of CMV transmission via blood product transfusion in high-risk patients such as HSCT recipients. One landmark study in the 1980s identified that the use of CMV-seronegative blood

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products significantly reduced the absolute risk of CMV infection by 21% in CMV-seronegative HSCT recipients when compared with standard, non-leukoreduced blood product use [16]. The standard of care since then has been to provide CMV-seronegative blood products to HSCT recipients.

The introduction of universal leukoreduction of blood products has resulted in a move away from this standard in some transplant centers. In Canada, currently the practice is divided in that half of transplant centers continue to require CMV-negative blood products for allogeneic HSCT recipients (S. Couban, M. Seftel, R. Foley, D. Stewart, and J. Sepher, personal communication by Internet survey through the Canadian Blood and Marrow Transplant Group, June 2012). There have been conflicting reports as to whether leukoreduced blood products are as effective at preventing transfusion-transmitted CMV as CMV-negative products when transfusing high-risk patients. Bowden et al. [17] demonstrated through a randomized prospective study that bedside filtration of leukocytes, an older, arguably less effective, method of leukoreduction, was as effective as CMV-seronegative blood products in preventing transfusion-transmitted CMV infection in HSCT recipients. In contrast, a prospective cohort study found transfusion of each additional filtered RBC unit from CMV-positive blood donors was associated with a 32% increase in the odds of developing transfusion-transmitted CMV [18]. In addition to leukoreduction, PCR monitoring for CMV and effective pre-emptive therapy with ganciclovir make it more unclear if there remains a need to provide CMV-negative blood products to HSCT recipients. We sought to identify whether there is a difference in transfusion-transmitted CMV viremia and disease in patients receiving only leukoreduced versus CMV tested negative and leukoreduced blood products in HSCT recipients at The Ottawa Hospital.

METHODS

Patients and Transfusion Data

We performed an uncontrolled "before–after" study using prospectively collected institutional data. Patients included in the analysis were serologic CMV-negative adults aged 18 years or older who received an allogeneic HSCT from a CMV-negative donor at The Ottawa Hospital HSCT Program between October 1, 1999 and June 30, 2012. Patient demographics included age, gender, disease, graft type, graft source, related or unrelated match status, degree of HLA match, ABO blood group compatibility, and conditioning regimen. All patients provided consent for collection of relevant health information for clinical research. Data were analyzed in an anonymous fashion in accordance with approval of The Ottawa Hospital Research Ethics Board.

Transfusion data were collected from The Ottawa Hospital Blood Bank. CMV status of transfused units was obtained from Canadian Blood Services. As per Canadian Blood Services standards, all RBC and platelet products were leukoreduced at collection before component storage. The use of leukoreduced RBC and platelet products began at The Ottawa Hospital on September 3, 1999 (P. Lesley, Canadian Blood Services, personal communication). As of January 1, 2007, as per change in policy at The Ottawa Hospital, patients receiving an allogeneic HSCT received standard leukoreduced products that were not specifically tested for CMV. Hereafter throughout this study, patients who received only leukoreduced blood products are referred to as leukoreduced, whereas patients who received leukoreduced blood products that were tested to be CMV negative are referred to as CMV negative. All patients in this study received irradiated (25 Gy) blood products.

Clinical Outcomes

The primary outcome was CMV viremia, defined as PCR positivity, evaluated in all patients who met the above criteria. CMV testing was done on a weekly basis after neutrophil and platelet engraftment after transplantation until immune suppression was discontinued. CMV testing was performed using the Amplicor CMV monitoring assay (Roche Diagnostics, Branchburg, NJ). This assay has been used at our center since 1998. Amplicor is an automated PCR assay using an enzyme immunoassay detection system. The linear range of the assay is 400 to 100,000 copies per milliliter. The sensitivity and specificity of this Amplicor assay has previously been established as 96% and 98%, respectively [19]. CMV disease was defined as tissue biopsy–proven CMV or CMV seropositivity with signs and symptoms known to be associated with CMV, namely retinitis, ependymitis, hepatitis, esophagitis, colitis, and pneumonia [20].

Secondary outcomes were total length of stay in hospital; admission to the intensive care unit; time to neutrophil engraftment, defined as a neutrophil count $> .5 \times 10^9$/L for at least 3 days; time to platelet engraftment, defined as a platelet count $> 50 \times 10^9$/L with no platelet transfusions for 3 days; incidence of grades II to IV acute graft-versus-host disease (GVHD) at 100 days; incidence of chronic GVHD, reported for patients surviving at least 100 days; 100-day nonrelapse mortality, defined as death in the absence of underlying disease within the first 100 days; RBC transfusion requirements during the 30 days after the marrow infusion; and platelet transfusion requirements during the 30 days after marrow infusion. GVHD was graded using standard published criteria [21].

Statistical Analysis

Baseline characteristics were described using measures of central tendency and dispersion where appropriate. For the comparison of proportions, chi-square or Fisher's exact test were performed. Times to engraftment and transfusion requirements were analyzed using Student's *t*-test. Continuous variables were analyzed using Wilcoxon rank test. All statistical analyses were carried out using SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

From October 1, 1999 to June 30, 2012, 166 patients were identified who met inclusion criteria. Eighty-nine patients received an HSCT before January 1, 2007, during the time when patients received leukoreduced and CMV-negative blood products. One hundred percent of patients in this cohort received CMV-negative blood products alone. Seventy-seven patients received an HSCT after this time, receiving only leukoreduced blood products.

The 2 groups did not differ in terms of age or gender (Table 1). There were no significant differences in diagnoses, graft type, graft source, conditioning regimen, or ABO compatibility between the 2 groups (Table 1).

There was no difference in the incidence of CMV viremia in those receiving CMV-negative versus leukoreduced blood products. CMV viremia was detected in 3 patients who received CMV-negative blood products (3.4; 95% confidence interval, .3% to 9.1%) and in 1 patient who received only leukoreduced blood products (1.3; 95% confidence interval, .7% to 9.5%; $P = .62$). Details of these 4 patients can be found in Table 2. All 4 patients received treatment with i.v. ganciclovir. Of the patients who developed CMV viremia, 2 developed suspected CMV disease. Both of these patients were transfused with CMV-negative blood products. One developed CMV viremia and acute respiratory failure requiring intensive care unit admission. This patient was concomitantly diagnosed with systemic *Aspergillus fumigatus* infection requiring antifungal therapy. The second patient also required admission to the intensive care unit for respiratory failure and was diagnosed with concomitant HHV-6 viremia. Both patients died of respiratory failure.

The mean length of stay for patients receiving CMV-negative versus leukoreduced only blood products was 41.5 and 46.8 days, respectively ($P = .47$). The proportion of patients who went to the intensive care unit was also not different in the 2 cohorts ($P = .10$). Time to neutrophil and platelet engraftment was not different between the 2 groups (Table 3, Figures 1 and 2). The incidences of overall (any grade) and severe (grades III to IV) acute GVHD were not different in the 2 groups ($P = .39$ and .13, respectively). The incidence of chronic GVHD was 22.5% in patients receiving CMV tested negative blood products versus 23.4% in patients receiving only leukoreduced blood products ($P = .52$). Overall

Table 1
Patient Characteristics

	CMV Negative (n = 89)		Leukoreduced (n = 77)		P
	n	%	n	%	
Gender (% female)	27	30.3	30	39.0	.24
Age (mean ± SD)	39.5 ± 13.4		43.5 ± 15.5		.07
Diagnosis					
ALL	12	13.5	10	13.0	.18
AML	19	21.4	27	35.1	
CML	14	15.7	2	2.6	
Hodgkin lymphoma	6	6.7	5	6.5	
Non-Hodgkin lymphoma	14	15.7	9	11.7	
MDS/MPN	9	10.1	10	13.0	
CLL	7	7.9	8	10.4	
Plasma cell disorders	2	2.3	2	2.6	
Other*	6	6.7	4	5.2	
Graft type					
PBSC	53	59.6	53	68.8	.21
BM	36	40.5	24	31.2	
Graft source					
Allo HLA match	46	51.7	30	39.0	.19
Allo HLA match unrelated	36	40.5	42	54.6	
Allo HLA mismatched	7	7.9	5	6.5	
Conditioning regimen					
Myeloablative	70	78.7	53	68.8	.21
Nonmyeloablative	19	21.4	24	31.2	
ABO mismatch	44	49.4	39	50.7	.88
30-Day transfusion needs (mean ± SD; units)					
SDP	9.6 ± 11.8		8.8 ± 10.8		.43
RDP	3.3 ± 15.8		.5 ± 2.6		.01
RBC	9.0 ± 10.6		7.2 ± 7.7		.23

ALL indicates acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; CLL, chronic lymphocytic leukemia; PBSC, peripheral blood stem cells; BM, bone marrow; SDP, single-donor platelets; RDP, random donor platelets.
* "Other" includes hemoglobinopathy, inherited disorders of metabolism, and nonhematologic malignancies.

survival was not different between the 2 groups ($P = .37$, Figure 3). The 100-day mortality for patients receiving CMV-negative blood products versus patients receiving only leukoreduced blood products was 22.5% and 14.7%, respectively ($P = .24$).

At 30 days, the number of packed RBC transfusions and single donor platelet units was not different ($P = .23$ and .43, respectively). There were, however, more transfusions of random donor platelets to patients receiving CMV-negative blood products versus those receiving leukoreduced-only transfusions (mean 3.3 versus .5 units, respectively; $P = .01$). Of the patients who received leukoreduced-only blood products, data were available on the CMV status of all units transfused until day 30 after HSCT for 63 patients (provided by Canadian Blood Services). The mean number of CMV-negative RBC units transfused was 6.4 ± 4.4, CMV-positive RBC units was 1.9 ± 1.4, CMV-negative platelet units was 3.7 ± 2.9, and CMV-positive platelet units was 4.1 ± 3.0. Patient D with CMV viremia (Table 2) who received leukoreduced-only blood products received 4 RBC units that were CMV negative, 2 RBC units that were CMV positive, 3 units of platelets that were CMV negative, and 11 units of platelets that were CMV positive within 30 days after HSCT.

DISCUSSION

Transfusion-transmitted CMV can be a potentially fatal consequence for HSCT recipients. Since the 1980s, CMV-seronegative transfusion products have been used in these patients to decrease CMV viremia and disease. It is unclear if this is still required in the era of universal leukoreduction of blood products in Canada. Our study demonstrated no difference in CMV viremia or disease in patients receiving CMV-negative leukoreduced versus leukoreduced-only blood products. This study suggests that in the age of universal leukoreduction in Canada, routine testing of blood products for CMV may not be warranted for HSCT recipients.

Our data are in accordance with reported results from other institutions. In a small study of 23 CMV-negative HSCT recipients from a CMV-negative donor, none of the patients developed anti-CMV antibodies after being transfused with CMV untested blood products, indicating that the risk of transfusion-transmitted CMV is close to zero with leukoreduction [22]. These data should be cautiously interpreted, however, because antibody formation in patients receiving an HSCT may be impaired due to immunosuppression. Although our incidence of CMV viremia was low with leukoreduction only (1.3%), the risk was still not zero. Although

Table 2
CMV-Positive Patients

Patient	HSCT Date	Transfusions	Time to CMV Detection*	Method of CMV Detection	CMV Disease	Outcome
A	Jan 2000	CMV negative	61	Blood - PCR	Pneumonitis	Respiratory failure, died day 190
B	Nov 2002	CMV negative	31	BAL - PCR	Pneumonitis	Respiratory failure, died day 35
C	Jul 2006	CMV negative	49	Blood - PCR	No	CMV negative after 3 wk of treatment
D	Jun 2007	Leukoreduced	32	Blood - PCR	No	CMV negative after 6 wk of treatment

BAL indicates bronchoalveolar lavage.
* Time to CMV detection reported in days after HSCT.

Table 3
Clinical Outcomes

	CMV Negative (n = 89)	Leukoreduced (n = 77)	P
Total LOS, d (mean ± SD)	41.5 ± 20.0	46.8 ± 32.0	.47
Patients who went to ICU, n (%)	15 (16.9)	21 (27.3)	.10
Days to neutrophil engraftment* (mean ± SD)	19.0 ± 5.9	19.3 ± 5.5	.65
Days to platelet engraftment* (mean ± SD)	32.5 ± 53.7	27.2 ± 22.9	.90
100-Day nonrelapse mortality, n (%)	13 (14.6)	9 (11.7)	.65
Acute GVHD (overall), n (%)	37 (41.6)	27 (35.1)	.39
Acute GVHD (severe), n (%)	9 (10.1)	14 (18.2)	.13
Chronic GVHD, n (%)	20 (22.5)	18 (23.4)	.52

LOS indicates length of stay; ICU, intensive care unit.
* Absolute neutrophil count >.5 × 10^9/L for at least 3 days; platelets >50 × 10^9/L with no platelet transfusions for at least 3 days.

it is possible that patients can develop CMV through means other than transfusion, these possibilities are less likely; therefore, we assume the transmission to be through transfusion. Seronegative donors still have the potential to pass CMV DNA to the transfusion recipient, especially in the window period after infection [23]. CMV testing in this circumstance will not recognize the donor as CMV positive because the antibody testing (which is standard practice for testing CMV in blood donors) will remain negative. This window period is believed to be days to weeks but has been reported to be as long as months [24]. This provides an explanation for the development of CMV viremia, albeit at a low incidence, even in patients receiving CMV-negative blood products. The inability of CMV serologic testing to detect window period donations provides further rationale for using standard leukoreduced blood products.

Similar to our study, CMV viremia in patients receiving leukoreduced blood products at the University of Michigan was not different in patients receiving CMV-negative versus CMV-untested transfusions [25]. In this study, however, most patients in the CMV-negative cohort received a mix of CMV-negative and CMV-untested blood products, making the results difficult to interpret. In our study, 100% of patients in the CMV-negative cohort received CMV-negative blood products.

Although transfusion-transmitted CMV was present in this study, the mortality related to CMV disease remained unclear. Two patients who were given CMV-negative and leukoreduced blood products developed presumed CMV disease based on CMV viremia and pulmonary symptoms thought to be related to CMV pneumonitis. In both cases, however, concomitant infections (*A. fumigatus* in 1 and HHV-6 in the other) may have contributed to the mortality observed in these patients. CMV disease was not observed in our cohort of patients receiving CMV-untested leukoreduced blood products after 2007.

Despite the small number of patients in this study, the results have important clinical implications. In a transplant center that has performed 491 allogeneic HSCTs during the time course of this study, 166 (33.8%) were CMV-negative donor and recipient pairs. The transfusion-transmitted CMV incidence was very low in patients receiving leukoreduced CMV-negative (3.4%) and -untested (1.3%) blood products, with no significant difference in nonrelapse mortality at 100 days after HSCT. In a moderately sized transplant center, the clinical impact of CMV testing was therefore negligible in the era of universal leukoreduction. If we estimate the incidence of CMV viremia to be 3% with CMV-untested blood products as observed in this study, a very large sample size would be needed in a randomized prospective trial to show a clinically relevant risk reduction. With an already low incidence of CMV viremia, this would be impractical and unlikely to change clinical practice.

There are limitations to our study that warrant attention, predominantly associated with our retrospective design. We attempted to minimize selection bias by including all

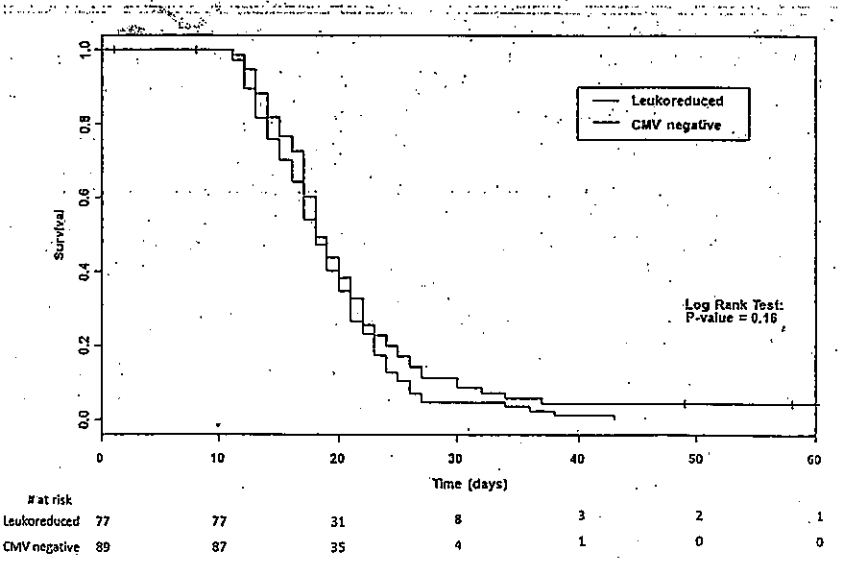

Figure 1. Neutrophil engraftment by transfusion group.

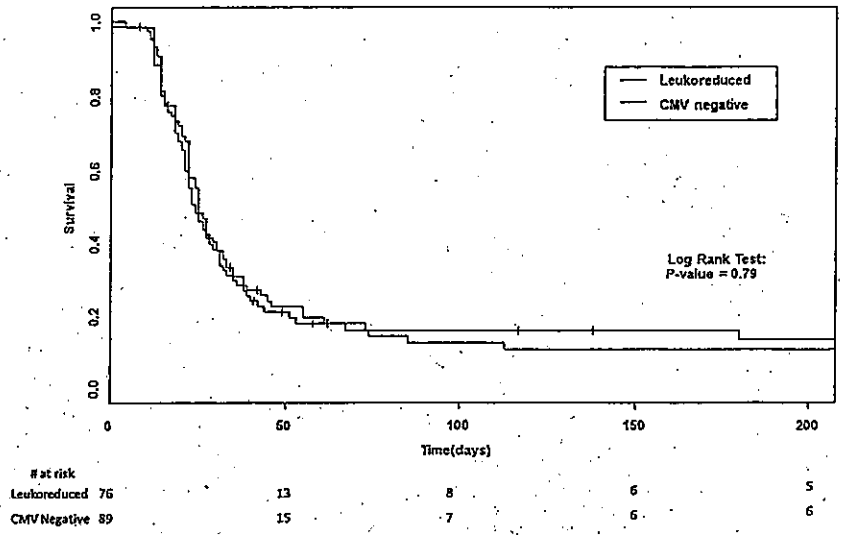

Figure 2. Platelet engraftment by transfusion group.

consecutive patients with CMV-negative patient–donor HSCTs at The Ottawa Hospital. Second, although our study provides acute and chronic GVHD rates, the types and duration of GVHD treatment were not captured. This could impact the risk of CMV viremia because patients receiving immunosuppressive treatment for GVHD would be at higher risk of CMV disease. Third, trends in transplantation practice may have influenced our results. General supportive care measures did not change during the study period. The practice of universal prophylaxis with acyclovir and the antivirals used for the treatment of CMV were unchanged throughout the study period, as was the policy of high-resolution HLA testing. Although no significant difference in baseline characteristics was identified, a higher proportion of patients with chronic myelogenous leukemia received CMV-negative leukoreduced blood products (15.7% versus 2.6%). This is due to the increasing use of tyrosine kinase inhibitors, eliminating the need for transplant in many of these patients.

The median age was nonsignificantly higher in the CMV-negative leukocyte reduced cohort, as was the use of peripheral blood as a stem cell source. Furthermore, random donor platelets were more commonly used in the earlier cohort of patients receiving leukoreduced CMV-negative blood products. This likely reflects a change in practice rather than a significant difference in the patient populations. Single-donor apheresis platelets have always been preferred to random donor platelets in HSCT recipients and have become more available in recent years. Our transplant center has also moved toward a more conservative threshold for platelet transfusions. These factors together contributed to the observation that patients receiving leukoreduced-only blood products received fewer random platelet donor transfusions. Despite these limitations, to our knowledge, this is the largest study to compare allogeneic HSCT recipients receiving CMV-negative versus CMV-untested blood products in the era of universal leukoreduction. A large randomized control trial would be impractical and would not

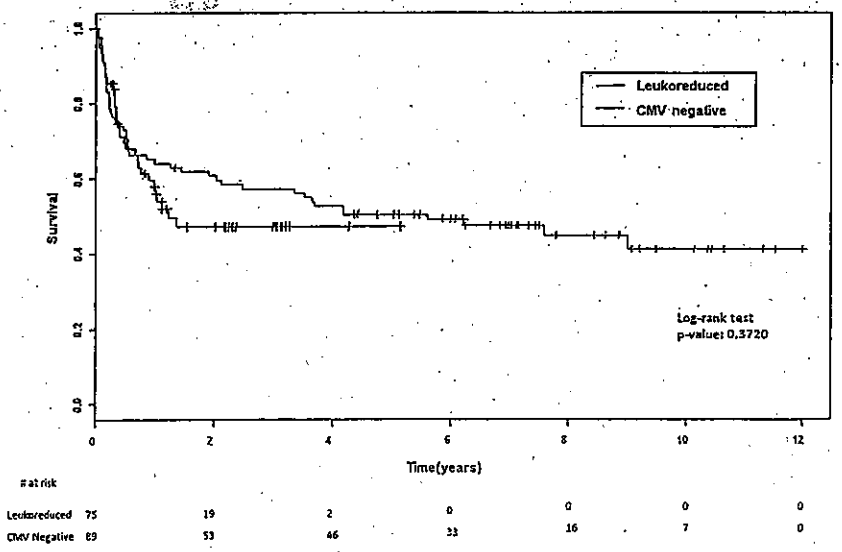

Figure 3. Overall survival by transfusion group.

likely show a difference in CMV viremia or disease that would impact clinical outcomes.

In the era of universal leukoreduction of blood products, this study demonstrates that testing for CMV-negative blood products is not needed in HSCT recipients. Our findings have implications for national health care resource utilization. Currently, about 47% of donors at Canadian Blood Services are individually tested for CMV. The cost of the medical supplies alone for CMV testing at Canadian Blood Services is estimated to be 745,000 CAD per year (G. Balkar, Canadian Blood Services, personal communication). HSCT recipients consume a significant number of blood products, and eliminating the need for CMV testing for this patient population nationwide could result in substantial cost savings.

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研究報告の概要

○日本の献血者におけるサイトメガロウイルス(CMV)抗体陽性率と高齢者におけるCMV DNAの高い検出頻度
背景: 日本におけるCMVの抗体陽性率と、全血液製剤が白血球除去されている現在のCMV輸血感染リスクは不明である。
研究デザイン及び方法: 性別と、10歳代から60歳代まで10歳毎に年齢で分類した12群の献血者から採取した全血2,400検体中のCMV特異的IgM及びIgG抗体を測定し、PCRで血球成分におけるCMV DNA検査を行った。
結果: 献血者の76.6%がCMV抗体陽性であった。抗体陽性率は、20歳代で58.3%、30歳代で73.3%であった。血球分画中のCMV DNAは、60歳代の献血者検体の4.3%、60歳未満の献血者の1.0%において検出された。さらに、DNA陽性検体の14%は血漿中にもDNAを含んでおり、そのような検体の5例中2例は60歳代の献血者由来であった。血漿中にCMV DNAを有する献血由来の白血球除去後の血漿製剤もまた、CMV DNAを含んでいた。
結論: 日本の20〜30歳代の献血者におけるCMV抗体陽性率は、過去15年間横ばいとなっている。潜伏CMVの再活性化は、若い献血者よりも高齢の献血者でより頻度が高かった。それらの一部は、血漿中に遊離CMV DNAを有し、白血球除去では取り除くことが出来なかった。血漿からCMV DNAが検出される献血におけるCMV輸血感染リスクの研究が必要である。

使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-LR「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
日本の献血者におけるCMV抗体陽性率を調査したところ、献血者の76.6%が抗体陽性であり、また60歳以上の献血者の4.3%からCMV DNAが検出され、そのような血漿の一部には白血球除去では取り除くことが出来ない遊離CMVが存在したとの報告である。	日本赤十字社では、保存前白血球除去した血液製剤のみを供給している。さらに、医療機関の要請に応じてCMV抗体(IgG及びIgM)が陰性であることを確認した血液製剤を供給している。今後もCMV感染に関する新たな知見等について情報の収集に努める。

Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors

Yasumi Furui,[1] Masahiro Satake,[2] Yuji Hoshi,[2] Shigeharu Uchida,[2] Ko Suzuki,[3] and Kenji Tadokoro[1,2]

BACKGROUND: The current prevalence of cytomegalovirus (CMV) in Japan and the risk of CMV transfusion transmission are unknown in the era of seronegative leukoreduced blood components.

STUDY DESIGN AND METHODS: We measured CMV-specific immunoglobulin (Ig)M and IgG in 2400 samples of whole blood collected from 12 groups of blood donors categorized by sex and age at 10-year intervals from their teens to their 60s. We also tested for CMV DNA using polymerase chain reaction in the cellular fractions of all samples.

RESULTS: We found that 76.6% of blood donors were CMV seropositive. The seroprevalences among donors in their 20s and 30s were 58.3 and 73.3%, respectively. We detected CMV DNA in the cellular fraction of 4.3% of samples from donors in their 60s and in 1.0% of samples from donors younger than 60 years. None of the 562 seronegative samples was DNA positive. Furthermore, 14% of DNA-positive samples also contained DNA in the plasma fraction, and two of five such samples were derived from donors in their 60s. Leukoreduced plasma components derived from donations with CMV DNA in plasma samples also contained a relevant amount of CMV DNA.

CONCLUSION: The seroprevalence of CMV among Japanese blood donors of child-bearing age has not changed over the past 15 years. Latent CMV becomes reactivated more frequently among elderly donors than among younger donors. A proportion of them have free CMV DNA in their plasma fraction, which could not be diminished by leukoreduction. The risk of transfusion-transmitted CMV infection in blood with plasma CMV DNA should be determined.

Human cytomegalovirus (CMV; *Human herpesvirus 5*) ubiquitously infects humans and persists in a latent form for long periods. It can cause asymptomatic infection in the general population or a mononucleosis-like syndrome or transient hepatitis in some healthy individuals. However, it can cause serious morbidity and mortality in immunocompromised hosts, and congenital or perinatal CMV infection causes developmental abnormalities in newborns. Morbidity can arise due to either primary infection or CMV reactivation. The transfusion of blood contaminated with CMV could be a source of primary infection in seronegative patients. Thus, CMV-safe blood components are typically required for transfusing seronegative patients who will undergo marrow or organ transplantation, patients with immunodeficiency syndrome, or premature infants. Blood facilities have implemented serologic screening of donated blood for CMV-specific immunoglobulin (Ig)G to mitigate the incidence of transfusion-transmitted CMV infection (TT-CMV) in such patients. This is conducted universally or in response to requests from physicians and has largely prevented TT-CMV infection.[1]

Leukoreduction using white blood cell (WBC) filters has been widely implemented in blood facilities to help reduce the side effects of residual WBCs in blood components such as febrile reactions or alloimmunization against WBC antigen. Leukoreduction under good

ABBREVIATION: TT-CMV = transfusion-transmitted cytomegalovirus infection.

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manufacturing practices could also abrogate the transmission of WBC-associated virus such as CMV, Epstein-Barr virus, or human T cell leukemia virus. Thus, leukoreduced blood components have been advocated as an alternative to transfusion for patients at risk for CMV when seronegative blood is unavailable, although whether leukoreduced blood is as safe as seronegative blood in terms of TT-CMV risk remains a matter of debate.[2-5]

Breakthrough cases have been attributed to transfusion with CMV-seronegative, but CMV DNA–positive blood that might have been donated during a window period, namely, the preseroconversion viremic phase of acute infection.[6] This could justify using leukoreduced blood to avoid transfusion with blood obtained during window periods that serologic screening could miss.[7] Thus, seronegative leukoreduced blood components are currently regarded as the safest strategy to prevent TT-CMV. However, Ziemann and colleagues[8] recently reported that up to 2.9% of plasma derived from donors during the window period contains CMV DNA. Because leukofiltration might not efficiently remove free CMV from the plasma fraction, this would pose another TT-CMV risk that could not be overcome by combining the two strategies.

We screened blood samples (n = 2400) donated equally by male and female volunteers of all age categories using serologic assays and nucleic acid amplification testing (NAT) to assess the risk of CMV transmission in Japan, particularly through transfusion with leukoreduced and seronegative blood components. We established a national prevalence and demographic trend for CMV infection over a range of donor ages and found no blood samples that were both viremic and seronegative. We also found that the frequency of CMV DNA positivity was higher in samples from elderly than from younger donors.

MATERIALS AND METHODS

Blood samples

We sequentially selected whole blood samples at the Japanese Red Cross Tokyo Blood Center in November 2010, where whole blood and blood samples were collected from five prefectures around the greater Tokyo metropolitan area. The samples were allocated to 12 groups according to donor sex and age at 10-year intervals from the 20s to the 60s and from age 16 to 19 years. Each of the 12 categories comprised 200 blood samples. Whole blood collected into tubes containing ethylenediaminetetraacetic acid was separated by centrifugation, during which the separation media rose to the interface between the plasma and the cellular fraction and formed a hard gel. We could thus keep them frozen until use without the two fractions becoming mixed. The plasma fraction was analyzed by CMV serology and CMV NAT. After removing the remaining plasma and interface gel, the top portion of the cellular fraction was suspended in the same volume of phosphate-buffered saline for DNA extraction.

CMV serology assays

We tested CMV-specific IgG and IgM antibodies using automated microparticle enzyme immunoassays (EIAs) and an immunochemical automated analyzer (AxSYM CMV-G and CMV-M, Abbott Laboratories, Abbott Japan, Tokyo, Japan).

DNA extraction

We extracted DNA from the cellular fraction of blood samples using the automated DNA purification kits (QIAsymphony SP and QIAsymphony DNA Midi kits, Qiagen, Tokyo, Japan) according to the protocol provided by the manufacturer (DNA Blood 1000). The input and output sample volumes were 1200 and 200 μL, respectively. Plasma DNA was likewise extracted from samples that were positive for DNA in the cellular fraction using a virus and bacteria detection kit (QIAsymphony Midi kit, Qiagen) with its accompanying protocol (Virus Cellfree 1000). The input and output sample volumes were 1.0 mL and 60 μL, respectively.

CMV NAT

We detected CMV DNA using TaqMan PCR and an sequence detection system (ABI PRISM7900HT, Applied Biosystems, Tokyo, Japan) and artus CMV TM PCR kits (Qiagen) according to the manufacturer's instructions.

We also prepared an in-house TaqMan PCR to detect CMV DNA. This system amplifies a 58-bp fragment of the UL83 gene that encodes phosphorylated 65-kDa proteins (pp65). The forward and reverse primers were 5'-TGCC ATACGCCTTCCAATTC-3' and 5'-TGGCTACGGTTCAG GGTCA-3', respectively. The TaqMan probe, 5'-CGGT AGATGTCGTTGGC-3', was labeled with a reporter dye (6-carboxyfluorescein, FAM) at the 5' end and a minor groove binder at the 3' end. The amplification reagent was supplied with a probe PCR kit (QuantiTect, Qiagen). Each reaction mixture comprised 30 μL of master mix and 20 μL of extracted DNA (equivalent to 120 μL of original sample). The thermocycling protocol comprised 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The nucleic acid concentration was calculated by measuring the absorbance of the extracted DNA at 260 nm.

A validation study for PCR sensitivity included NATtrol NATCMV-0004 (ZeptoMetrix, Buffalo, NY) as the external reference CMV for both PCR analyses. The reference solution was serially diluted in 5% bovine serum albumin (BSA) and portioned into small tubes for PCR analysis over a period of 4 days. We tested CMV concentrations five times daily for each PCR procedure, for a total

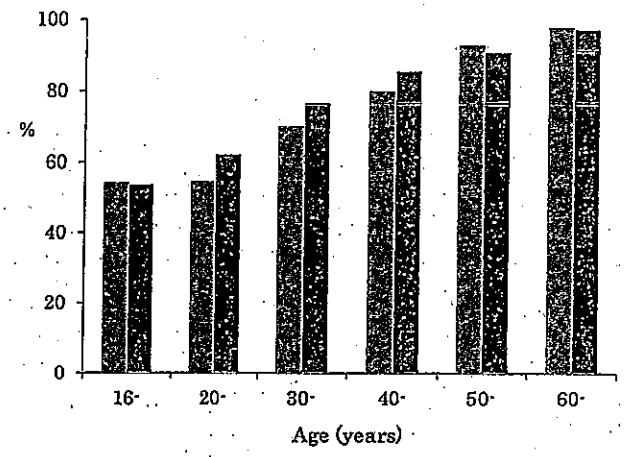

Fig. 1. Age distribution of CMV-specific IgG prevalences in (▨) men and (■) women.

of 20 replicates at each concentration. We then calculated the 95 and 50% limits of detection for each PCR using probit analysis. Correlation study between the reference solution and first World Health Organization international standard (NIBSC 09/162) revealed that 32.3 genome equivalents/mL (geq/mL) was equivalent to 1 IU/mL. Samples in which the PCR results were ambiguous were further analyzed using nested PCR targeting the UL139 sequence as described by Bradley and colleagues[9] with the modification for DNA polymerase (KAPA DNA polymerase, Nippon Genetics, Tokyo, Japan).

To adjust the amount of CMV DNA for the number of WBCs in the sample, we estimated the number of Exon 5 sequences of CD81 in specimens using real-time PCR.[10] CD81 was chosen as a marker of WBCs as it is present with two haploids in a cell. Amounts of CMV DNA are described as geq per 6.0×10^5 WBCs (geq/PBL unit) in this study. The lowest limit of quantitative CMV DNA detection was 40 geq/mL before adjustment for WBC numbers.

Statistical analysis

Data were analyzed using computer software (SSRI, Excel Statistics, Version 8, Social Survey Research Information, Tokyo, Japan; for Windows, Microsoft Excel 2007, Tokyo, Japan). Significance was determined using the chi-square test and t test. p values of less than 0.05 were considered significant.

RESULTS

We initially examined the prevalence of anti-CMV among Japanese blood donors. Figure 1 shows the prevalence of specific IgG among the age categories. The prevalence exceeded 50% even in male and female teenagers and steadily increased over time to reach nearly 100% in their 60s. Although not significant, the prevalence tended to be higher in females than in males aged from the 20s to the 40s. The increase in the prevalence was the highest between the 20s and 30s (15%; combined for both sexes) and gradually decreased with age to 5.8% between the 50s and 60s. The mean prevalence in the six age categories was 76.3%. The overall CMV prevalence adjusted for an assumed population with the age distribution of Japanese blood donors (Japanese Red Cross data, 2010) was 76.6%. The IgM prevalence was higher among females than males between the ages of 16 and 39 years ($p < 0.05$, Table 1). Seven donors were IgM positive and IgG negative, and four of them were teenagers.

We next examined the presence of CMV DNA in the cellular fraction of 2400 whole blood samples. A validation study showed that the 95 and 50% limits of CMV DNA detection for artus CMV TM PCR were 41.6 and 5.3 geq/mL, respectively, and those for the in-house PCR were 29.6 and 5.4 geq/mL, respectively (Table 2). Only samples that were positive for at least two PCR analyses including nested PCR targeting the UL139 sequence were defined as CMV DNA positive. We identified 37 samples that were positive for CMV DNA in the cellular fraction (Table 3). Four other samples were positive for only one PCR analysis and were defined as DNA indeterminate. Table 4 shows the relationship between DNA positivity and the serostatus of the specific antibody. We found DNA positivity in six (6.6%) of 91 samples that were both IgM and IgG positive and in 31 (1.8%) of 1740 that were only IgG positive. Although the samples that were positive only for IgM did not contain any that were DNA positive, the frequency of DNA positivity was significantly higher in six (6.12%) of 98 samples that were IgM positive with or without IgG than in those that were positive only for IgG ($p < 0.03$). Viral load was significantly higher in CMV DNA–positive samples that were both IgM and IgG positive (mean, 670 geq/PBL unit) than in those that were only IgG positive (170 geq/PBL unit, $p < 0.03$, t test). Notably, none of the 562 samples that were both IgM and IgG negative was also DNA positive.

Table 5 compares the distribution of 37 DNA-positive samples with age categories. The frequency of DNA positivity was significantly higher (17/400, 4.3%) among donors in their 60s than in any other age category (0.8%-1.3%, $p < 0.03$) from the teens to the 50s or the combined age category (1.0%, $p < 0.03$) from 16 to 59 years. The range of viral load in the 37 DNA-positive samples was between less than 40 and 3.4×10^3 geq/PBL unit (mean, 250 geq/PBL unit; median, 80 geq/PBL unit). The difference in viral load in the samples between donors aged less than 60 years (mean, 310 geq/PBL unit) and those in their 60s (mean, 170 geq/PBL unit) was not significant. The presence of DNA in the plasma fraction was further investigated in these 37 samples. Five (13.5%) of them were plasma DNA positive with a viral load between less than

TABLE 1. Prevalence of CMV-specific IgM among blood donors*

	Male		Female		Total	
Age (years)	Positivity	Percent	Positivity	Percent	Positivity	Percent
16-19	6	3.0†	13 (4)	6.5†	19 (4)	4.8
20-29	5 (1)	2.5†	15	7.5†	20 (1)	5.0
30-39	5 (1)	2.5†	13	6.5†	18 (1)	4.5
40-49	8	4.0	10	5.0	18	4.5
50-59	5 (1)	2.5	3	1.5	8 (1)	2.0
60-69	6	3.0	9	4.5	15	3.8
Total	35 (3)	2.9	63 (4)	5.3	98 (7)	4.1

* Numbers of donors positive only for specific IgM are shown in parentheses.
† IgM prevalence significantly higher among female donors than among male donors (16-19, 20-29, and 30-39 years); chi-square test ($p < 0.05$).

40 and 5.8×10^3 geq/mL (median, 170 geq/mL). These five were scattered across age categories with two being in their 60s. One sample obtained from a teenaged donor was IgM and IgG positive and the other four were positive only for IgG. We identified two more samples from donors in their 60s that were DNA positive with only one PCR analysis.

We interdicted three components of fresh-frozen plasma that had CMV DNA in the plasma fraction. All of them were derived from whole blood that was leukoreduced before storage. We also detected CMV DNA in all three plasma components. One component donated by the IgM- and IgG-positive teenaged male donor contained 9.7×10^3 geq/mL CMV DNA and the other two components that were positive only for IgG from donors in their 60s contained 1.9×10^2 and 1.6×10^3 geq/mL CMV DNA.

DISCUSSION

We investigated the prevalence of CMV among Japanese blood donors categorized by sex and age at 10-year intervals. The more than 50% prevalence of CMV infection among individuals aged between 16 and 19 years is in contrast with the approximately 30%[11] prevalence in other developed countries. The increase in the prevalence (15%) between donors aged in their 20s and 30s implies that young adults become infected with CMV at a rate of 1.5% per annum. This is similar to the annual rate of 1.69% observed between 1994 and 1999,[12] implying that the risk of CMV infection among females of child-bearing age that is directly related to symptomatic fetal CMV infection has not changed over the past 15 years. The reason for the sustained high prevalence in Japan is unclear, but prolonged breast-feeding and communal child care practices in Japan probably influenced the rates in younger donors. The prevalence in Japan has become almost maximal after the age of 60 years, which contrasts with the continuous lifelong primary infection found in other developed countries.[11] The CMV seroconversion rate (1.33%)[11] among German blood donors aged 30 to 35 years is close to the 1.5% rate of increase described above. However, care must be taken in comparing the present results with those of the German study because our results were generated from a cross-sectional study whereas the German findings were obtained through longitudinal follow-up of seronegative donors. Although insignificant, the prevalence in females tended to increase sooner than in males, a finding that is consistent with the higher prevalence of specific IgM in younger females than in younger males.[13,14]

We detected CMV DNA in the cellular fraction of 1.7% (41/2400) of all, or 2.2% (41/1831) of the seropositive, samples with or without specific IgM. This frequency was comparable to those reported by Greenlee and colleagues[15] and Roback and colleagues.[16] We found CMV DNA more frequently in samples that were IgM positive than in those that were only IgG positive (6.12% vs. 2.0%, $p < 0.03$), indicating that active CMV replication occurs more frequently during acute primary infection that is often accompanied by IgM positivity. None of the samples from the group of seven donors that was positive only for IgM was CMV DNA positive. This is reasonable because Ziemann and coworkers[17] detected CMV DNA only in 10% of 148 primary seroconverted blood donors. At that rate we would be unable to identify a single DNA-positive individual in our study population. The same authors showed that CMV DNA levels peak during the late phase of primary infection in newly seropositive donors.[8] Although whether a rationale exists for introducing screening for specific IgM in addition to IgG remains to be determined,[18] the chemiluminescence tests for CMV currently applied by the Japanese Red Cross detect only IgG. Although we have discussed seroprevalence and its relationship with the presence of DNA by interpreting IgM positivity as representing primary infection, reactivity for CMV-specific IgM measured by EIAs must be considered with caution. Several articles have reported frequent nonspecific reactions[19,20] and suggest including Western blot analyses or IgG avidity assays to ensure reactivity. Because of the small plasma volume of most of the donor samples, we were unable to apply these analyses. Thus, the above findings and our interpretations based on categories by IgM positivity might be inconclusive and require further investigation.

We found no DNA-positive samples among 562 that were seronegative, suggesting that the likelihood of donating DNA-positive blood during the window period[7] is very low in Japan. This finding is similar to that described by Roback and coworkers,[16] who found no CMV DNA positivity among 514 healthy, seronegative blood donors. However, these findings do not allow underestimation of

TABLE 2. Determination of sensitivity of two PCR systems by replicate testing and probit analysis

PCR	Ref* (geq/mL)	D 1	D 2	D 3	D 4	Total	95% LOD (geq/mL)	50% LOD (geq/mL)
Artus CMV	1	1/5	1/5	1/5	1/5	4/20	41.6	5.3
	5	5/5	3/5	2/5	5/5	15/20		
	10	5/5	5/5	4/5	4/5	18/20		
	50	5/5	5/5	5/5	5/5	20/20		
In house (UL83)	1	0/5	1/5	0/5	1/5	2/20	29.6	5.4
	5	5/5	3/5	5/5	4/5	17/20		
	10	5/5	5/5	4/5	5/5	19/20		
	50	5/5	5/5	5/5	5/5	20/20		

* NATtrol as CMV reference was diluted in 5% BSA.
artus = artus CMV TM PCR kits (Qiagen); D = day; in house (UL83) = in-house PCR targeting CMV UL83 sequence; LOD = limit of detection.

TABLE 3. CMV DNA–positive samples and PCR procedures

PCR results	Number of samples
UL83 positive and artus positive	29
UL83 repeatedly positive	2
artus repeatedly positive	2
UL83 positive and UL139 positive	3
artus positive and UL139 positive	1
Indeterminate*	4
Negative	2359
Total	2400

* Positive in only one PCR analysis.
artus = artus CMV TM PCR kits (Qiagen); UL139 = PCR targeting CMV UL139 sequence; UL83 = in-house PCR targeting CMV UL83 sequence.

the risk of TT-CMV caused by transfusion with window period–derived blood components because we did not focus on blood samples obtained at the time of acute primary infection when CMV replication is most likely to be active. In fact, Ziemann and colleagues[9] found that two (2.9%) samples were DNA positive among 68 plasma samples obtained from final seronegative donations during the course of seroconversion. Collectively, a risk of TT-CMV related to window period donation exists but the frequency seems very low.

The frequency of detecting CMV DNA was 4.3% among donors in their 60s, compared with 1.0% (0.8%-1.3%) in the population aged from 16 to 59 years. Considering that the specific IgG prevalence has already peaked by age 60 years in Japan, the notion that the DNA-positive individuals in their 60s were nonimmune to CMV and emitted CMV virions during the course of primary CMV infection is inconceivable. Latent CMV more likely became reactivated in those elderly individuals. The reactivation of CMV in elderly persons is thought to represent "immunosenescence" caused by chronic CMV infection.[21,22] The current concept of immunosenescence in relation to CMV infection is that terminally differentiated memory T cells accumulate with ageing in the limited peripheral "immunologic space," which causes a progressive decline in the generation of naive T cells that protect against new pathogens. In addition, a considerable portion of the accumulated memory T cells were specific for CMV.[23-25] Thus, CMV infection is considered a driving force or risk biomarker for the constitution of a skewed peripheral T-cell repertoire. Despite conflicting results and ideas about epidemiology and immunologic mechanisms, the clinical impact of the CMV infection on individuals who are not immunocompromised has remained a central question.[22] Whether or not all persons with CMV infection acquire skewed T-cell phenotypes with aging, the kinds of socioeconomic or physical factors that facilitate this process, and when this process starts to compromise the immune system should be addressed. We established statistical evidence of CMV reactivation occurring in the peripheral blood of voluntary blood donors in their 60s. Viral load did not significantly differ between donors in their 60s and those aged less than 60 years. Blood donors in Japan are supposedly healthy individuals who have all been qualified by questionnaires and consultation with physicians. Our results therefore suggest that CMV reactivation is a constitutional event in CMV carriers and starts to occur during the sixth decade of life, although the possibility remains that donors positive for CMV DNA recently might have had specific illnesses or behaviors that are related to CMV reactivation. The findings of animal experiments suggest that lytic viral reactivation is necessary to establish the peripheral T-cell repertoire skewed for CMV.[26] Stowe and colleagues[27] detected CMV in 57% of urine samples from elderly individuals (66 to 83 years) but in none of those from younger individuals (25 to 55 years). This would also suggest that CMV reactivation occurs more frequently among elderly, than younger, individuals, although they did not detect CMV in any blood samples from both groups. However, this might have resulted from the small sample size studied (11 elderly individuals compared with 400 aged ≥60 years in this study). The rather clear cutoff of the reactivation frequency between the 50s and 60s is reminiscent of a Swedish study[28] showing increased 10-year mortality

TABLE 4. Association between CMV serostatus and CMV DNA positivity

Serology status	Number of samples	DNA+ (n)	Ratio (%)	Viral load Mean/median Range (geq/PBL unit)
IgM–/IgG–	562	0	0	
IgM+/IgG–	7	0	0*	
IgM+/IgG+	91	6	6.6*	670/62†
IgM–/IgG+	1740	31	1.8*	<40-3400 170/80† <40-920
Total	2400	37	1.5	

* DNA positivity ratio significantly higher in IgM+ than in IgM–/IgG+ samples (6 [6.1%] of 98 vs. 31 [1.8%] of 1740); chi-square test ($p < 0.03$).
† Viral load in IgM+/IgG+ samples significantly higher than that in IgM–/IgG+ samples; t test ($p < 0.03$).
DNA+ = DNA positive.

TABLE 5. Age distribution of CMV DNA positivity in cellular and plasma fractions*

Age (years)	Cellular fraction DNA+	Cellular fraction Ratio (%)	Plasma fraction, DNA+
16-19	4 (2)	1.0	1 (1)
20-29	5	1.3	1
30-39	5 (1)	1.3	0
40-49	3 (2)	0.8	0
50-59	3	0.8	1
16-59 total	20 (5)	1.0	3 (1)
60-69	17 (1)	4.3	2†
Total	37 (6)	1.5	5

* Numbers of donors specifically positive for both IgM and IgG are shown in parentheses.
† In addition to these two samples, two others from donors in their 60s were positive for one PCR analysis.
DNA+ = DNA positive.

rates among individuals with immune risk profiles at the age of 65 years but not at the age of 55 years.

We identified five (13.5%) samples that were positive for CMV DNA in the plasma fraction of 37 blood samples that contained CMV DNA in the cellular fraction. This result is comparable to the report by Ziemann and coworkers[8] in which 44% of blood samples from 82 recently seroconverted donors were CMV DNA positive in the plasma fraction. Drew and colleagues[6] also reported that three of 384 samples obtained from 192 seroconverted blood donors contained low plasma levels of CMV DNA. We quantified CMV DNA levels in three plasma products derived from donations that had CMV DNA in plasma samples. The DNA levels (1.9×10^2 to 9.7×10^3 geq/mL) were comparable to those measured in the plasma samples. Because all blood components including apheresis-derived plasma components are leukoreduced in Japan, this finding indicated that leukofiltration cannot reduce levels of free CMV DNA in the plasma fraction.

The identification of blood donations with plasma fractions containing CMV DNA raises concerns about the safety of blood components. The residual risk of TT-CMV under the current blood program that applies both seroscreening and universal leukoreduction could be focused on blood with plasma viremia that is provided during the window period because plasma viremia might not be appropriately managed by leukofiltration. This leads to the notion of Ziemann and colleagues[8] that leukoreduced components would be safer when obtained from seropositive donors at least 1 year after seroconversion. This is also based on the finding that plasma viremia has barely been detectable among donors who remain seropositive for more than 1 year. However, our study showed that a proportion of latently infected individuals presents with free CMV DNA in plasma fractions. Free CMV DNA in plasma could not be effectively diminished by prestorage leukoreduction, which was verified by assays of leukoreduced plasma products. Therefore, the strategy suggested by Ziemann and colleagues, while eliminating window period–related risk, might generate another risk associated with blood containing free plasma CMV DNA that is provided mainly by elderly donors. Although we identified only two samples from donors in their 60s that were plasma DNA positive, one of them related to a plasma product containing 1.6×10^3 geq/mL CMV DNA, a viral load that was comparable to that for window period donation provided by the teenager (9.7×10^3 geq/mL). Moreover, we found two other samples with possible plasma DNA among donors in their 60s, although they were DNA positive only for a single PCR analysis, suggesting low DNA concentrations. Whereas we found that whole blood CMV DNA positivity among donors in their 60s was 4.3%, that identified in an elderly US population with a mean age of 84.5 years was 42.3%.[29] Thus, since the frequency of whole blood CMV increases dramatically after 60 years, we can speculate that the frequency of plasma CMV also increases with age. In this context, serious problems could arise in countries that accept donors over 70 years of age if seropositive donations are accepted for transfusion into patients at risk. Although leukoreduced blood components have been advocated as an alternative when seronegative blood is not available, they might carry a higher risk of TT-CMV

than seronegative blood, which might partly explain higher TT-CMV frequency among patients transfused with leukoreduced blood compared with seronegative blood.[2,4]

Further study is required to compare possible TT-CMV risks between persistently seropositive blood that might contain free CMV virions due to reactivation and seronegative blood that might incidentally contain such virions due to window period donation. Ziemann and coworkers concluded from a recent extensive study of more than 22,000 samples that TT-CMV risk is essentially comparable between window period donations among seronegative donors and donations with reactivation among long-term seropositive donors.[30] Before assessing the TT-CMV risk in Japan, the degree to which window period donation constitutes the blood donor population will need to be determined. Other basic issues also need to be resolved. Because we used techniques involving DNA amplification but not viral culture for plasma study, whether CMV DNA identified in plasma with this technique constitutes replication-competent virions remains unknown.[31,32] Whether plasma products containing free CMV virions is infectious through blood transfusion also needs to be resolved.[13,33] Whether blood components containing CMV virions possibly derived from reactivation in latently infected blood donors are as infectious as those derived from donations provided during acute primary infection also requires investigation. The clinical relevance of CMV neutralizing antibody that can be found in latently infected individuals also must be considered. Finally, the minimal infectious dose of CMV virions acquired through blood transfusion should be determined for each type of blood component.

In conclusion, the seroprevalence of CMV among a Japanese population of blood donors was 76.6%. The prevalence among donors in their 20s and 30s has not changed over the past 15 years. We detected CMV DNA in 1.7% of 2400 samples. None of the 562 seronegative samples was DNA positive. We detected CMV DNA more frequently in blood from donors aged in their 60s than from younger donors. Among DNA-positive samples, 14% contained DNA in the plasma fraction, and this frequency might be higher among donors in their 60s than younger donors. CMV DNA persists in a portion of seropositive blood even after prestorage leukoreduction and leukoreduced blood without seroscreening might not be as safe as seronegative blood in terms of TT-CMV risk. The risk of TT-CMV in blood with detectable CMV DNA in the plasma fraction should be determined.

CONFLICT OF INTEREST

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研究報告の概要

背景：サイトメガロウイルス（CMV） DNA は、高い頻度で新規に抗体陽性となったドナーの血漿に検出される。過去の CMV 感染ドナーからの白血球除去血液製剤は、初期感染ドナーからの輸血後 CMV 感染（TT-CMV）を回避することができたかもしれない。しかし、血清陰性ドナーのウィンドウ期間の献血の発生率と比較して、長期抗体陽性ドナーの再活性化の有病率についてのデータはない。従って、リスクのある患者のための最適な輸血戦略は不明である。

研究デザインと方法：22,904 の献血からの全血サンプルで CMV DNA について検査し、CMV DNA 陽性の献血は、1）抗体陰性ドナー、2）新規抗体陽性ドナー、3）長期抗体陽性ドナー からの献血として分類した。

結果：21 人のドナー（0.09%）は、再現性がある CMV DNA 陽性であった。全血中の CMV DNA の検出頻度および濃度は、抗体陰性および長期抗体陽性ドナーで同等であった。全血中の CMV DNA に対する再現性のない陽性結果は、長期的な陽性ドナーでより頻度が高かった。血漿の CMV DNA の低濃度は、2 人の抗体陰性ドナーと 1 人の長期抗体陽性ドナーで検出可能であった。しかしながら、全血および血漿の両方で最も CMV DNA の高い濃度は、新規抗体陽性ドナーで発見された。

結論：抗体陰性ドナーにおけるウィンドウ期間中の供血頻度と長期抗体陽性ドナーにおける再活性化、更にはそれらのドナーからの全血および血漿サンプル中の CMV DNA 濃度は同等であった。従って、新規抗体陽性ドナーからの献血のリスクが増加する一方で、抗体陰性、長期抗体陽性の両方のグループからの血液製剤は、TT-CMV のリスクがある患者のために使用することができるだろう。

報告企業の意見	今後の対応
サイトメガロウイルス（Cytomegalovirus：CMV）は、ヘルペスウイルス科（Herpesviridae）ベータヘルペスウイルス亜科（Betaherpesviridae）に属する、直径約 180nm のエンベロープを有する球状の 2 本鎖 DNA ウイルス［学名：human herpes virus-5（(HHV-5)］で、種特異性が強く、ヒト以外の動物には感染しない。万一、原料血漿に CMV が混入したとしても、Bovine herpes virus（BHV）をモデルルウイルスとしたウイルスクリアランス試験成績から、本剤の製造工程において不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

使用上の注意記載状況・その他参考事項等

代表としてテタノブリン IH 静注 250 単位の記載を示す。

2. 重要な基本的注意

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

The impact of donor cytomegalovirus DNA on transfusion strategies for at-risk patients

Malte Ziemann, David Juhl, Siegfried Görg, and Holger Hennig

BACKGROUND: Cytomegalovirus (CMV) DNA is frequently detected in plasma of newly seropositive donors. Selection of leukoreduced blood products from donors with remote CMV infection could avoid transfusion-transmitted CMV infections (TT-CMV) due to primarily infected donors. However, there are no data about the prevalence of reactivations in long-term seropositive donors compared to the incidence of window period donations in seronegative donors. Therefore, the optimal transfusion strategy for at-risk patients is unclear.

STUDY DESIGN AND METHODS: Whole blood samples from 22,904 donations were tested for CMV DNA, and CMV DNA–positive donations were categorized as donations from 1) seronegative donors, 2) newly seropositive donors, and 3) long-term seropositive donors.

RESULTS: Twenty-one donors were reproducibly CMV DNA–positive (0.09%). Frequency of detection and concentration of CMV DNA in whole blood were comparable for seronegative and long-term seropositive donors. Nonreproducibly positive results for CMV DNA in whole blood were more frequent in long-term seropositive donors (0.16% vs. 0.01%, $p < 0.01$). Only low concentrations of CMV DNA in plasma were detectable in two seronegative donors and one long-term seropositive donor. Highest concentrations of CMV DNA in both whole blood and plasma, however, were found in newly seropositive donors.

CONCLUSION: Prevalences of window period donations among seronegative donors and reactivations among long-term seropositive donors, as well as the CMV DNA concentration in whole blood and plasma samples from these donors, are comparable. Therefore, blood products from both groups could be used for patients at risk for TT-CMV, while those of newly seropositive donors seem to bear an increased risk.

In immunocompetent subjects, cytomegalovirus (CMV) usually causes asymptomatic or mild mononucleosis-like infections leading to lifelong latency with possible reactivations.[1] Besides the usual transmission by direct person-to-person contact, blood transfusions are an additional source of infection in risk groups like low-birthweight newborns or patients receiving allogenic stem cell transplants, in whom CMV contributes to increased morbidity and mortality.[2,3] Transfusion-transmitted CMV infections (TT-CMV) have traditionally been explained by transmission of latently infected white blood cells (WBCs).[4] The risk of TT-CMV can be reduced by approximately 92% by WBC reduction of blood components,[5] but even after implementation of leukoreduction, "breakthrough" infections persist with rates as high as 1% to 3% of transfused high-risk patients.[5]

This residual risk could be due to residual WBCs despite leukoreduction or to not-cell-associated CMV found during primary CMV infections and reactivations of latent infections.[6,7] Donors with latent CMV infections or during the late phase of primary CMV infections can be recognized by detection of CMV antibodies. Donations from those donors can be avoided by selection of seronegative blood products for patients at risk for TT-CMV.[2] On the other hand, selection of seronegative blood products increases the proportion of donations from donors with primary CMV infection before development of CMV-specific antibodies (window period donations). The selection of blood products from latently infected donors (e.g.,

ABBREVIATIONS: gB = glycoprotein B; TT-CMV = transfusion-transmitted cytomegalovirus infections.

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donors who have been seropositive for at least 1 year) has been proposed as alternative transfusion strategy for at-risk patients.[8] A further alternative might be provision of blood products tested negative for CMV DNA, even if the currently available nucleic acid testing (NAT) assays require testing of single or only few pooled plasma samples to reach sufficient sensitivity.

To determine which transfusion strategy is associated with the lowest risk of TT-CMV, the incidence and intensity of viremia, or detection of CMV DNA as surrogate variable, must be compared for seronegative and long-term seropositive donors. As both window period donations and reactivations of latent CMV infections are rare events, large numbers of donors must be studied. Ideally, not only free CMV in plasma, but even cell-associated CMV is measured to account for possible transmission of CMV infections by residual WBCs. To our knowledge, no study examining whole blood samples from a significant number of donors has been performed. Therefore, we conducted this study examining whole blood samples from 22,904 consecutive donations for CMV DNA.

MATERIALS AND METHODS

Donors

All donors from the donation site Lübeck of the Institute of Transfusion Medicine at the University Hospital of Schleswig-Holstein between March 9 and October 26, 2011, who donated on a Monday, Tuesday, or Wednesday, were included in the study. After inclusion of 22,265 donors on October 26, only the first 94 donors on each second Tuesday were included until March 8, 2012. Between March 9, 2011, and March 8, 2012, a total of 23,112 donations were included in the study.

Preparation of blood products

Red blood cell (RBC) units were produced from whole blood donations and leukoreduced by in-line filtration with a leukoreduction filter (Asahi Sepacell Pure RC, Fenwal, Inc., Lake Zurich, IL). Pooled platelet (PLT) concentrates were produced by the buffy coat method and leukoreduced by in-line filtration with a leukoreduction filter (Asahi PLX5, Fenwal, Inc.). Fresh-frozen plasma (FFP) was produced without filtration from whole blood donations after separation of the buffy coat. Apheresis PLTs were produced as leukoreduced PLT concentrates without filtration by apheresis (Amicus, Fenwal, Inc.; or Trima Accel Terumo BCT, Lakewood, CO).

Detection of CMV DNA

DNA from 200-µL aliquots of whole blood samples was isolated within 18 hours after sampling using a total DNA isolation kit (ZR-96 Quick-gDNA, Zymo Research, Irvine, CA). An aliquot of 20 µL of DNA, corresponding to 80 µL of whole blood, was tested for CMV glycoprotein B (gB)–specific sequences using a highly sensitive TaqMan polymerase chain reaction (PCR) as described elsewhere.[6] Samples with invalid internal control or positive results were retested twice. The presence of CMV DNA was confirmed by reproducibly positive PCR results. For all donors with at least one positive result for CMV DNA in whole blood, 1-mL plasma samples were also tested for CMV DNA with a 95% detection limit of 12 IU CMV/mL.[9]

To determine the 95% detection limit for CMV DNA in whole blood samples, semilogarithmic dilutions of the First WHO International Standard for Human CMV for Nucleic Acid Amplification Techniques (NIBSC code 09/162, National Institute for Biological Standards and Control, Potters Bar, UK) containing between $10^{4.5}$ and 10^{9} IU/mL CMV strain AD169 were investigated. Twenty-four samples of each concentration were processed in three consecutive TaqMan PCR runs. The 95% detection limit was calculated by means of probit analysis to be 235 IU/mL whole blood (confidence interval, 154-511 IU/mL, Table 1). This limit of detection corresponds to a CMV DNA content of leukoreduced blood products of less than 120 IU/product, if it is assumed that all DNA is contained in WBCs, blood donors have WBC counts of at least 2×10^{9} WBCs/L, and the maximum WBC content of leukoreduced blood products is approximately 10^{6} WBCs/unit.

Quantification of CMV DNA–positive samples was carried out by a standard curve derived from these validation experiments as well as measurements of eight samples with 10^{5} IU/mL. As CMV DNA concentrations below 300 IU/mL whole blood or 30 IU/mL plasma could not be quantitated reliably for single samples, those values were used only for statistical evaluation. The CMV DNA concentration for 208 of 23,112 samples (0.9%) could not be evaluated, because there were no valid PCR runs and the sample volume was too low for additional measurements.

TABLE 1. Limit of detection and probability of positive PCR results for serial dilutions of CMV DNA*

CMV DNA standard (IU/mL whole blood)	Number of valid samples†	Observed responses	Probability of positive results (%)
31,600	24	24	100
10,000	24	24	100
3,160	24	24	100
1,000	24	24	100
316	24	23	99.6
100	22	17	51.0
31.6	24	4	21.3
10	24	2	14.6
3.2	24	3	12.8
1	24	2	12.2

* The probit analysis resulted in a 95% limit of detection of 235 IU CMV DNA/mL whole blood.
† Negative samples with invalid internal control were excluded.

Detection of CMV antibodies

Antibodies against CMV were tested in all previously seronegative or untested donors by a generic chemiluminescent microparticle enzyme immunoassay against AD169-coated microparticles (Architect Anti-CMV IgG assay, Abbott GmbH & Co. KG, Wiesbaden, Germany). For CMV DNA–positive samples, additionally the recom-Blot CMV Western blot (Mikrogen GmbH, Neuried, Germany) was performed. The Western blot contained the recombinant antigens CM2 and IE1 (similar to the nonstructure proteins p52 and IE1 "immediate-early protein"), p65 and p150 (similar to the tegument proteins pp65 and pp150), and gB1 and gB2 (resembling the membrane glycoprotein gB). Band intensity was categorized into "very weak," "weak," "well visibly," and "very strong intensity" according to the manufacturer's instructions.

Classification of CMV DNA–positive donors

Additional follow-up samples and/or plasma samples obtained at previous donations and stored at below –30°C were analyzed to classify CMV DNA–positive donors into the following categories:

1. Donors within the window period of primary CMV infection: CMV DNA–positive donors with a negative result of the generic CMV antibody test for the DNA-positive sample and detection of CMV antibodies in follow-up samples.
2. Donors in the early seropositive phase of primary CMV infection: CMV DNA–positive donors with a positive result of the generic CMV antibody test for the DNA-positive sample and negative results for CMV antibodies in previous samples (primarily seropositive donors).
3. Donors with presumed latent CMV infection: CMV DNA–positive donors with positive results of the generic CMV antibody test for the DNA-positive sample and for a sample drawn at least 1 year before.

Twelve donors were excluded from this comparison: Two were seronegative donors, in whom neither CMV antibodies nor CMV DNA were detected in follow-up samples drawn on Days 127 and 232 and on Days 35 and 54, respectively, after the CMV DNA–positive sample. One of these donors had only one positive PCR result for CMV DNA in whole blood; the other showed two positive and two negative PCR results for CMV DNA in whole blood. Both donors tested negative for CMV DNA in plasma. The other 10 excluded donors were seropositive donors without earlier samples to distinguish between presumably latent and primary infection. Two of these donors were CMV DNA positive in plasma.

Estimation of the maximum CMV DNA content in leukoreduced blood products

The CMV DNA concentration in whole blood due to infected WBCs was estimated as:

(CMV DNA concentration in whole blood) – (CMV DNA concentration in plasma) × (1 – hematocrit).

Assuming the donors' WBC count to be at least 2×10^9 WBCs/L, the maximum CMV DNA content of leukoreduced blood products was calculated by the formula

(CMV DNA concentration in whole blood due to infected WBCs) / (2×10^9 WBCs/mL) × (maximum content of WBCs per unit).

To determine the maximum concentration of residual WBCs in leukoreduced blood products, data from the mandatory quality assessment analysis were used: 497 RBC units, 378 FFP units, 79 buffy coat–derived pooled PLT concentrates, and 97 PLT apheresis concentrates were randomly selected from the routine production during the study period and tested for residual WBCs by flow cytometry (DNA-Prep Coulter REF 6607055, Beckman Coulter, Krefeld, Germany).

The maximum CMV DNA content of blood products due to DNA in plasma was calculated assuming a plasma content of 18 mL for RBCs, 300 mL for plasma units, and between 60 and 300 mL for PLT concentrates (depending on whether PLTs are stored in plasma or in storage solution).

Statistical analysis

Unless stated otherwise, means are calculated as arithmetic means ± standard deviation (SD). Calculations were assisted by database and statistical programs (Excel, Microsoft Corp., Redmond, WA; SPSS, Inc., Chicago, IL). Differences between groups were examined using the chi-square test for categorical data.

RESULTS

Detection of CMV DNA

Twenty-one of 22,904 donors were reproducibly CMV DNA positive (0.09%). High concentrations of CMV DNA of at least 1000 IU/mL whole blood or 100 IU/mL plasma were reached in four primarily seropositive donors (0.02% of all donors). CMV DNA concentrations in both seronegative donors or long-term seropositive donors were below these limits. While latently infected donors accounted for the majority of CMV DNA–positive whole blood samples, CMV DNA in plasma was mostly detected in primarily seropositive donors. Detailed results according to the donors' stage of CMV infection are shown in Table 2 and Fig. 1.

Antibodies against gB were detectable in three of nine primarily seropositive donors. Plasma samples of these three donors were negative or only weak positive (less

TABLE 2. Detection of CMV DNA and antibodies according to the donors' stage of CMV infection*

	Window period of primary CMV infection	Early seropositive phase of primary CMV infection	Latent CMV infection
Whole blood samples			
CMV DNA reproducibly positive	4	6	6
Only a single positive result for CMV DNA	0	3	12
Median CMV DNA concentration (range), IU/mL	400 (3 samples <300, 500)	500 (4 samples <300, 500, 600, 2000, 2000, 4000)	<300 (15 samples <300, 300, 600, 800)
Plasma samples			
CMV DNA reproducibly positive	2	7	1
Only a single positive result for CMV DNA	0	1	0
Median CMV DNA concentration (range), IU/mL	40 (<30, 70)	50 (3 samples <30, 50, 90, 150, 400, 700)	0 (<30)
Prevalence (%) of CMV antibodies in CMV DNA–positive donors	0	100	100
Prevalence (%) of antibodies against gB in CMV DNA–positive donors	0	33	100 (one weak)

* Twelve CMV DNA–positive donors had been excluded from this comparison: Two seronegative donors without seropositive follow-up samples and 10 seropositive donors without samples drawn at least 1 year before the CMV DNA–positive sample to distinguish between presumably latent and primary infection. Five of the 12 excluded donors were reproducibly CMV DNA positive, and seven had only a single positive result for CMV DNA.

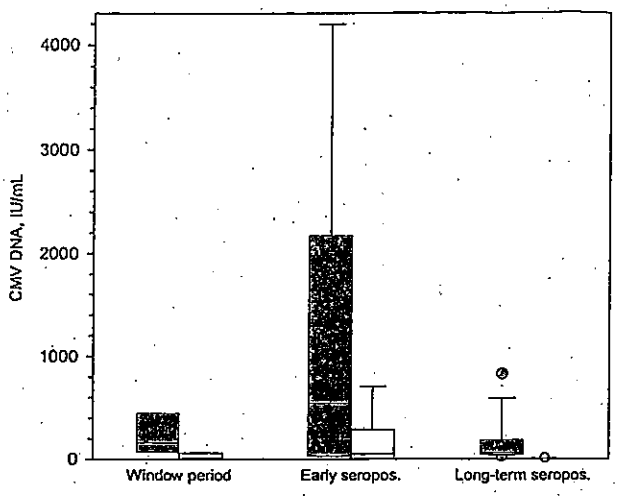

Fig. 1. CMV DNA concentrations in whole blood and plasma samples according to the donors' stage of CMV infection. CMV DNA concentrations in whole blood and plasma samples are given for donors in the window period of their primary CMV infection, primarily seropositive donors in the early seropositive phase of primary CMV infections, and long-term seropositive donors with presumed latent infection. The boxes represent the 25th and 75th percentiles with the median DNA concentration marked by a thin line. Error bars correspond to the 5th and 95th percentiles. As there were only four CMV DNA–positive window period donations, no error bars can be calculated for this group. (■) Whole blood; (□) plasma.

than 30 IU/mL) for CMV DNA. The only long-term seropositive donor with evidence of CMV DNA in plasma showed weak CMV antibodies (59 arbitrary units per mL, only weak against p150 and gB1, but not against gB2).

Estimation of the maximum CMV DNA content in leukoreduced blood products

The median concentration of residual WBCs was 0.01×10^6 or less for all tested kinds of blood products. The maximum amount of residual WBCs in leukoreduced RBCs was 0.15×10^6 per unit, in FFP 0.08×10^6 per unit, in buffy coat–derived pooled PLT concentrates 0.05×10^6 per unit, and in PLT apheresis concentrates 1.4×10^6 per unit. The rate of CMV DNA–positive donations according to different transfusion strategies and the maximum CMV DNA content in leukoreduced blood products are shown in Table 3. Whole blood samples from seronegative donors were less frequently CMV DNA-positive than samples from unselected donors, long-term seropositive donors, or plasma NAT–negative donors ($p < 0.01$). This effect was caused by a lower proportion of donors with a single positive PCR result only, while the frequency of donors with reproducibly positive PCR results was not significantly different.

TABLE 3. Impact of transfusion strategies on the risk of TT-CMV

Transfusion strategy (in addition to leukoreduction)	Unselected donors	Seronegative donors	Long-term seropositive donors	Plasma NAT-negative* donors
Donations available for at-risk patients	22,904	13,236	7,303	Approx. 22,700†
CMV DNA in whole blood				
CMV DNA positive	21 (0.09%)	5 (0.04%)	6 (0.08%)	10 (0.04%)
Only a single positive PCR result	22 (0.10%)	1 (0.01%)‡	12 (0.16%)	18 (0.08%)
Maximum CMV DNA concentration, IU/mL	4,000	600	800	600
CMV DNA in plasma				
CMV DNA positive	12 (0.05%)	2 (0.02%)	1 (0.01%)	0§
Only a single positive PCR result	1	0	0	0
Maximum CMV DNA concentration, IU/mL	700	70	<30	<12
Maximum CMV DNA content in leukoreduced blood products				
Maximum CMV DNA content due to infected WBCs, IU/unit				
RBC units	300	50	60	60
Plasma units	200	30	30	30
PLT units (filtered buffy coat)	100	20	20	20
PLT units (apheresis)	3,000	400	600	600
Maximum CMV DNA content due to DNA in plasma, IU/unit‖				
RBC units	10,000	1,000	<600	<200
Plasma units	200,000	20,000	<9,000	<4,000
PLT units	40,000-200,000	4,000-20,000	<2,000-<9,000	<700-<4,000

* Limit of detection 12 IU/mL plasma.
† Estimated, because not all donations were tested for CMV DNA in plasma.
‡ $p < 0.01$ versus all other strategies. $p = 0.001$ versus unselected donors.
§ $p = 0.001$ versus unselected donors.
‖ Assuming a plasma content of PLT units of between 60 and 300 mL/unit and 18 mL plasma per RBC unit.

DISCUSSION

Examining 22,904 blood donations over a period of 1 year, CMV DNA was detected more frequently in long-term seropositive donors compared to seronegative donors. If only donations with reproducibly positive results for CMV DNA in whole blood were considered, however, the difference between seronegative and long-term seropositive donors was not significant.

Usually, nonreproducibly positive PCR results are considered to be false positive. Of course, there might be false-positive results in the current study as well. The ambiguous results for CMV DNA in whole blood samples from two seronegative donors, whose follow-up samples all tested negative for both CMV DNA and CMV antibodies, for example, are likely to be caused by false-positive PCR results. The significant difference in the rate of nonreproducibly positive PCR results according to the donors' serostatus, however, cannot be explained by false-positive results, as all samples were processed in order of donation irrespective of the donors' serostatus.

Another explanation for nonreproducibly positive PCR results is a DNA concentration well below the limit of detection of the applied PCR. This is illustrated by the decreasing rate of positive results for serial dilutions used to calculate the limit of detection (see Table 1). As latently infected monocytes are supposed to contain two to 13 genome equivalents of CMV per cell,[4,10] nonreproducibly positive PCR results might even be caused by single latently infected cells from seropositive donors. Only one long-term seropositive donor was positive for CMV DNA in plasma, suggesting a reactivation of a latent CMV infection.

Peak CMV DNA concentrations in window period donations and donations from long-term seropositive donors were below 1000 IU/mL whole blood or 100 IU/mL plasma. As shown earlier for plasma samples, maximum concentrations for CMV DNA were reached in newly seropositive donors in the late phase of their primary CMV infection.[6] Also viable CMV in body fluids was detected approximately 2 weeks after the first seropositive sample in a study of CMV-vaccinated and nonvaccinated subjects by Zhang and colleagues.[11] This delayed peak has been explained by the hypothesis that primary CMV infections caused by direct person-to-person contact first lead to a localized lytic infection cycle, for example, in the nasopharyngeal mucosa, initiating production of antibodies against nonstructure and tegument proteins.[9] After an interval of days to several weeks, this local infection is followed by viremia during which the virus spreads to other organs, like the kidneys. Concurrently, antibodies against glycoproteins of the viral envelope, like gB, are induced, which probably contribute to termination of the viremic phase.[9] The duration of viremia in immunocompetent subjects seems to be short in most subjects, but even cases with prolonged viremia up to several months have been reported.[12]

If no CMV-specific testing is conducted (e.g., as recommended by the German guidelines) and all donations are used for at-risk patients, significantly more donations

are CMV DNA positive (0.19% instead of 0.05%, $p < 0.01$), and approximately sevenfold maximum CMV DNA-concentrations are reached compared to blood products from seronegative donors. Even leukoreduced blood products might contain up to 3000 IU CMV DNA per unit due to residual WBCs and up to 200,000 IU/unit due to free CMV in plasma.

In unselected leukoreduced blood products and units from seronegative donors, more than 90% of the CMV DNA content is caused by CMV DNA in plasma. On the other hand, even leukoreduced blood products from donors tested CMV DNA negative with a sensitive plasma NAT screening might contain up to 600 IU CMV per product due to infected WBCs. Even such a low number of residual, latently infected WBCs could be more efficient in transmitting CMV infections than a high viral load in plasma, because CMV infections might be transferred more easily by direct cell-to-cell contact than by free virus. Viable CMV during primary CMV infections of immunocompetent subjects, for example, was undetectable in plasma samples, but found in six of 110 WBC samples in the studies of Zanghellini and colleagues[13] and Revello and coworkers.[14]

Direct measurement of viable virus in CMV DNA–positive blood products by viral cultures would have also been desirable in the current study, but the detected concentrations of CMV DNA were below the limit of detection of common culture techniques. Nevertheless, CMV DNA in plasma correlates with CMV disease[15] and is recommended as a surrogate variable in monitoring at-risk patients for active CMV infections.[16] Presence of CMV DNA in whole blood might as well correspond to systemic viremia, but as discussed previously, low concentrations of CMV DNA in whole blood might even be due to latent virus. To our knowledge, there are no data about the infectious dose of CMV, but even low concentrations might be infectious for immunocompromised patients.

Whether breakthrough infections actually occur despite use of leukoreduced blood products is debated controversially: Thiele and coworkers[17] did not detect a single case of TT-CMV in a cohort of patients with stem cell transplantation, but were only able to include 23 CMV-seronegative donor–patient pairs. Statistically, no case of TT-CMV in 23 patients corresponds to a 95% probability of breakthrough infections occurring in less than 13%. This risk of TT-CMV was calculated to be 6.5% by Wu and coworkers,[18] based on three cases of possible TT-CMV among 46 CMV-seronegative and DNA-negative transfusion recipients. However, there was no definite proof from donor follow-up that the infections were transfusion associated. For low-birthweight infants (at most 1500 g), Josephson and colleagues[19] are currently conducting a study to determine the incidence of TT-CMV and to evaluate the effectiveness of transfusing leukoreduced blood products from seronegative donors.

Also from the current study, the optimal transfusion strategy for at-risk donors cannot be determined clearly: The classic strategy of seronegative blood products has the lowest risk of transfusing cell-associated CMV. This effect is only significant, however, if even very low numbers of infected cells are taken into account. Window period donations with prominent viremia were not detected in the current study, which is in accordance with current findings that the peak CMV DNA concentration is reached after seroconversion.[9]

As expected, NAT-negative blood products showed the lowest risk for free CMV in plasma, but this difference reached significance only for comparison with unselected donors. Blood products from long-term-seropositive donors contained potentially neutralizing antibodies against membrane proteins like gB, which might impair infection of at-risk patients by low concentrations of CMV.

Whole blood NAT would be an ideal option to detect both cell-associated and free CMV, but this approach is challenging, especially as a low limit of detection is required. To weigh the advantages and disadvantages of the investigated transfusion strategies, detailed knowledge would be necessary about 1) the comparative infectivity of low concentrations of cell-associated versus free CMV and 2) the influence of neutralizing antibodies on the infectivity of low concentrations of CMV.

Until these facts are known, avoidance of blood products from newly seropositive donors remains the most important goal in preventing residual TT-CMV despite leukoreduction. As shown earlier for plasma samples,[6] newly seropositive donors also showed highest concentrations for CMV DNA in whole blood, while no potentially neutralizing antibodies were detectable in donors with high CMV DNA concentration yet. Therefore, these products should imply the highest risk of transmitting CMV to at-risk patients. Avoiding these blood products is possible by provision of seronegative blood products, CMV NAT–negative blood products, or blood products from long-term seropositive donors. In remaining cases of suspected TT-CMV, both the serostatus of all implicated donors (seronegative, newly seropositive, long-term seropositive) and the CMV DNA concentration in stored plasma samples should be determined to gather further knowledge on which donors confer the lowest risk for TT-CMV.

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CONFLICT OF INTEREST

None of the authors declares a conflict of interests.

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研究報告の概要

ヒトパルボウイルス B19（B19V）は、子供の伝染性紅斑（リンゴ病）、溶血性貧血患者の骨髄無形成発症や慢性赤芽球癆、妊婦の胎児水腫や胎児死亡、免疫不全患者の持続性貧血の様なヒトにおける種々の疾患を引き起こす。B19V は主に呼吸系、血液および血漿由来製剤を介して感染するが、特に（高ウイルス量であるが症状のない献血者からの B19V DNA の高レベルで汚染された）凝固因子製剤も感染性であることが示された。B19V はノンエンベロープウイルスの病原体で、有機溶媒/界面活性剤（S/D）処理の様な不活化方法は血漿中のウイルスの低減に効果がない。
汚染された血液および血漿由来製剤を介した B19V 感染のリスクを低減するために、日本赤十字社（JRC）血液センターは 1997 年 9 月に、受容体媒介赤血球凝集反応分析（RHA）で B19V スクリーニングを実施した（これは既に北海道では試験的に 1996 年 4 月に実施されていた）。RHA は、グルタルアルデヒドを固定した赤血球（RBC）が酸性条件（pH 5.2-5.8）の下で RBC 膜上のグロボシドを介して B19V 粒子を経て凝集する B19V 抗原検出方法である。これはプール血漿中の B19V の汚染レベルを低減した。
最近では、血漿分画製剤の全てではないが、いくつかの製剤の製造用プール血漿は、ヨーロッパにおいて B19V DNA が 4LogIU/mL を超えないことを要求されていた、一方米国においては全てのプール血漿に推奨されていた。この様な理由で、製造業者は B19V DNA を検出し、そして高ウイルスを排除するために、核酸増幅検査（NAT）を用いてミニプール血漿をスクリーニングしている。RHA が輸血用血液と同様に血漿分画製剤用プール血漿中の B19V ウイルス量の低減に大いに貢献していたにもかかわらず、RHA の検出限界（約 10LogIU/mL）は血漿分画プール中の B19V DNA 量を 4LogIU/mL を超えないことを確実にするには不十分である。2008 年、感度を向上するために、JRC 血液センターは化学発光酵素免疫測定法（CLEIA-B19V）による B19V 抗原スクリーニングを導入した。CLEIA-B19V は 2 段階サンドイッチ測定法で、異なる特性を持つ B19V に対してマウスモノクローナル抗体（MoAbs）が使われた。
B19V は 3 つの遺伝子型 1、2 と 3 に分類されている。遺伝子型 1 と 2 は米国、ヨーロッパおよび他の西欧諸国で発見された。一方遺伝子型 3 は大部分はサハラ以南のアフリカと南アメリカに限定された。北および中央ヨーロッパでは、遺伝子型 2 株がしばしば高齢者の組織サンプルで見つかっている。遺伝子型 2 は 1970 年代までこの領域の人達に流行した先祖（原型）のウイルスであることが報告された、それから遺伝子型 1 と置き換えられた。3 つの主要な遺伝子型に加えて、ベトナム人患者で遺伝子型 1 株の別の亜型（1b）とブラジル及びヨーロッパで遺伝子型 3 株の亜型（3b）が報告されている。ヨーロッパ薬局方および米国食品医薬品局（FDA）の生物製剤評価研究センターは、B19V の 3 つの遺伝子型全てが NAT 手法によって検出されなければならないことを勧告した。現在利用できる市販キットと社内 B19V DNA 測定法は、最近確認した遺伝子型 2 と 3 の検出、あるいは定量が出来なかった。そのため、報告は日本人献血者の B19V 遺伝子型解析のための調査で利用できなかった。そして CLEIA-B19V が B19V に関するこれらの勧告に対応する代替アプローチでありえるかどうかは不明であった。従って、我々は CLEIA-B19V による全ての遺伝子型の検出の可能性を研究し、日本人の献血からプールした分画用原料血漿中の B19V DNA 量を測定し、過去 15 年間の日本の北の島、北海道の献血者の間の B19V の遺伝子型を調査した。

材料と方法
B19V 抗原のための献血者スクリーニング
JRC 血液センターで、全ての献血は血清学的検査と NAT 検査でスクリーンされた。血清陽性サンプルは、NAT 検査の間の交差汚染のリスクを低減するために 20 プールの 3 回 NAT スクリーニングから除外された（図．1）。2008 年以降、全ての個別献血は CL4800 検査システム（富士

使用上の注意記載状況・その他参考事項等

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

レビオ）のCLEIA-B19Vを用いてB19V抗原をスクリーニングした。CLEIA-B19Vは2段階サンドイッチ測定法で、異なる特性を持つB19Vに対するマウスMoAbsを使用した。B19Vは抗体の2つのグループの間にサンドイッチされた：1つは磁気粒子と結合し、もう一方はアルカリ性ホスファターゼ（ALP）でラベルした。B19V抗原の量は、ALPによって加水分解した3(-2′-Spiroadamantane)-4-methoxy-4(-3″-phosphoryloxy)-phenyl-1,2-dioxetaneの化学発光により測定した。1.0以上、或いは同等のCLEIA-B19V値（カットオフ指標［COI］）を持つサンプルは、B19V抗原に陽性であると判断した。繰り返し反応性サンプルは、輸血用血液および血液成分、そしてJRCにより更に製造する血漿用から除外した。
後述するように、北海道のCLEIA-B19V陽性サンプルは、更なる研究のためにB19V用の広く使われているリアルタイム・ポリメラーゼ連鎖反応（U-PCR）によってB19V DNAを分析した。
北海道の献血者から、2008年8月から2009年5月まで無作為に抽出した合計682のCLEIA-B19V陰性サンプルは、B19V用U-PCRによるB19V DNAも分析した。

（省略）

結　　果

北海道におけるCLEIA-B19Vによる献血者スクリーニング

2008年2月から2011年9月まで（44箇月）の間、CLEIA-B19Vでスクリーニングした北海道の1,035,560の献血の内、101（0.01%）のU-PCR陽性サンプルを含む417（0.04%）が繰り返し陽性であった（図2）。101のB19V DNA陽性献血の内、67（66%）は2010年12月から2011年6月までの流行期の間に検出された（図2）。316（0.03%）のCLEIA-B19V陽性、B19V DNA陰性献血は、61人の繰り返し陽性ドナーを含む171人のドナーからのものであった。
B19V DNAとCLEIA-B19Vの両方が陽性であるドナーサンプル（n=101）は、抗B19V抗体の存在に従って感染の4期に分けられた（暫定的にI群、II群、III群およびIV群と名づけた）：

- I群（感染初期）-抗B19Vに対するIgMとIgG陰性；
- II群（感染の初期から中期）-IgM陽性とIgG陰性；
- III群（感染の中期から後期）-IgMとIgG陽性；
- IV群（感染の後期から末期）-IgM陰性、IgG陽性。

IV群のドナーの一人はB19V DNAの高レベルを示した（>8LogIU/mL；表1）。このドナーはIII群からIV群（末期でない）への過渡期にあると見なすことができた。IV群（ウイルス量<4LogIU/mL）の8つの献血は5人のドナーからのもので、その内の一つは4つの状況に関係した陽性献血である。
表．1に示したように、I群とII群の献血（n=63）は6LogIU/mLよりも大きな高B19 DNA量を示した、それとは逆にB19V-IgGとB19V-IgMが陽性であったIII群の献血（n=29）はI群とII群の献血よりもより低いウイルス量を示した。IV群の献血の大部分は低ウイルス量（<4LogIU/mL）を示した。
CLEIA-B19Vによって陰性であった682のサンプルの内、21（3.1%）は抗B19Vに対するIgGが陽性およびB19Vの低ウイルス量（<4LogIU/mL）であった。

遺伝子型パネルおよびドナーサンプルのＣＬＥＩＡ－B19V値とB19V DNA量の相関関係

図3Aは、CLEIA-B19V値（3つの測定値の平均）が各遺伝子型パネル部（MS1-MS3）のB19V DNA量に対応していることを示す。非常に直線的な相関は、近似値によってCLEIA-B19V値とB19V DNA量の間で見つかった（R2>0.99）。それぞれの遺伝子型パネルサンプルの回帰直線は特定の範囲に収束した。CLEIA-B19Vの感度は、各々の回帰直線がCLEIA-B19Vのカットオフ値（1.0 COI）と交差するB19V DNA量に基づき、約6.3LogIU/mL（遺伝子型1、6.23LogIU/mL；遺伝子型2、6.11LogIU/mL；遺伝子型3a、6.23LogIU/mL）と推測された。
図3Bは、CLEIA-B19V値がB19V DNA陽性サンプルのB19V DNA量に対応していることを示す。CLEIA-B19Vの最大値は2000のCOIでセットさ

ない。感染した場合には胎児への障害（流産、胎児水腫、胎児死亡）が起こる可能性がある。］

れる。IV群（ウイルス量<4LogIU/mL）の8つの献血を除いた、そして2000のCOIのCLEIA値を持つ40の献血は、53のサンプルを用いた回帰直線が直線的な相関（R2=0.87）および3つの遺伝子型のパネルを使用したのとほとんど同じ感度（約6.4LogIU/mL）を示した。

北海道の献血者間のB19Vの系統分析

北海道の198のB19V DNA陽性献血のDNA配列は例外なく遺伝子型1に分離した（図4）。遺伝子型1の198株は、（暫定的に亜群A、B、Cと名づけた）少なくとも3つのマイナーな亜群に分けられた。亜群Bは1996年から2011年の間に恒常的に検出された、それとは逆に亜群Aはこの間に早く消えた、そして亜群Cは2008年にだけ現れた（図4.）。3つの亜群の間で、有意差は北海道における年齢、性別および地理的分布で観察されなかった（データは示さない）。各亜群の株の間のヌクレオチド変動は、0.9%から3.2%までの範囲であった。各亜群の変動は1.6%未満であった。2007年と2011年の伝染性紅斑の2つの最近のアウトブレイクの間にドナーからのそれぞれの主要株は2つの異なるクラスタ中に現れた、しかし全て亜群Bに分離され、高いヌクレオチド類似性を示した（>98.4%、図.4）。

プール血漿中のB19Vの汚染レベル

表2は日本全国のドナーからの原料血漿プールのバッチにおけるB19V DNAの量を示す。RHAによりスクリーニングした血漿からなる原料血漿の2,118のバッチの内、365（17.2%）バッチは4LogIU/mL以上のB19V DNAで汚染されていた。対照的に、CLEIA-B19Vによる血漿スクリーニングの開始で、プール血漿中のB19V汚染は減少した。CLEIA-B19Vスクリーニングへ完全切り替えの後、（最新の流行期から得られた殆ど全ての血漿を含んだ）全772のプール血漿中のB19V DNA量のレベルは、米国およびヨーロッパの推奨レベルである4LogIU/mLを超えなかった。

議　　論

公式化した第一回WHO国際B19V遺伝子型パネル（遺伝子型1、2と3a）のためのNATによる以前に評価した幾つかの中間体ウイルス系統を使用およびB19V DNA陽性ドナーサンプルで6.4LogIU/mLの時、我々はCLEIA-B19Vの感度が約6.3LogIU/mLであると推測されることを示した。FDAは、血漿由来製剤の製造業者がB19V DNAの存在を検出するために以下の手順を実行するよう最近勧告した：

1）全ての血漿由来製剤用B19V NATは、ウイルスの全ての既知遺伝子型を検出しなければならない。

2）製造用プール中のB19V DNAのウイルス量は4LogIU/mLを超えない。

2008年、JRCの血清学的B19Vスクリーニングは、感度を改善するために従来の凝集方法（RHA）からCLEIA方法に切り替えられた。血漿分画製剤用の原料血漿は日本の無報酬の自発的ドナーからの血漿からなり、CLEIA方法によるスクリーニングも全ての血漿分画製剤の安全性に貢献する（図1）。しかしながら、JRC血液センターのCLEIA-B19Vスクリーニングが FDAの2つの勧告に応じた代替方法でありえるかどうかは明らかでなかった。

FDAによる最初の勧告に関しては、B19Vの3つ遺伝子型全ては単一血清型を構成することが報告されている、一方これら3つのウイルス型は全てのゲノム上で約10%～15%異なる。CLEIA-B19Vは抗原－抗体反応に基づく検出システムであるので、ほぼ等しい感度でB19Vの3つの遺伝子型全てを検出することは合理的である。FDAによる第2の勧告のために、我々は日本国中のドナーからプールした分画用の原料血漿中のB19V DNA量を測定した。CLEIA-B19Vスクリーニング、およびB19V陽性単位のその後の排除は、プール血漿中のウイルス量を著しく減らした（<4LogIU/mL）。従って、CLEIA-B19VはスクリーニングFDAの2つの勧告に対応することが分かった。

我々は、感染期に基づく4つの群の北海道のCLEIA-B19Vスクリーニングによって検出したB19V DNA-陽性献血を暫定的に分けた。Ⅰ群とⅡ群の献血は、非常に高いウイルス血症で、CLEIA-B19Vによって確実に検出されると考えられるB19V感染の初期から中期に対応した。特に流行期の間、高いウイルス量のB19V陽性血漿は、プールしたNATスクリーニングに関して交差汚染のより大きなリスクがある。もしB19V NATスクリーニングが導入されたなら、CLEIA-B19Vスクリーニングはプールした NATスクリーニング前のⅠ群とⅡ群の献血の様に高いウイルス量を持つB19V陽性血漿を排除することが可能であるという長所がある。非常に高いウイルス血症B19V陽性献血（Ⅰ群～Ⅲ群）は、2008年2月から2011年9月までの北海道の93の献血（0.009%）で検出された。オランダからの最近の報告では、高力価（>6LogIU/mL）のB19Vの陽性率は、2003年から2009年の間の全てのオランダの献血の0.006%(411/650万)であった。同様に、米国からの報告では、高力価(>6LogIU/mL)のB19Vの陽性率は2000年から2003年までで0.016%(2/12,529)であった。大規模な献血を用いたこれら最近の発生率は、北海道からの我々

のデータと非常に類似している。日本では、オランダの場合のように、B19V DNA陽性ドナーの増加と相関する症候性B19V感染の流行サイクルは、4年から6年の間隔で起こることが報告されている。北海道のB19Vの流行期の間、多くのB19V株が100%のヌクレオチド同一性を共有することが分かった。北海道と日本全国の間で殆ど同じ流行間隔にもかかわらず、類似の傾向が日本の他の地域で観察されるかどうかについて発見することに興味がある。

I群およびII群と対照的に、CLEIA-B19Vによる抗原検出はIII群サンプル中の抗体の過剰な存在で不安定である場合がある。事実、特にB19V DNAが7LogIU/mL前後の献血で、CLEIA-B19V値の相当な変動があった。このように、DNAの量が7LogIU/mL前後のその様な全ての献血はCLEIA-B19Vによって検出される可能性があるというわけではないが、CLEIA-B19Vの感度は約6.4LogIU/mLであると推測される。しかしながら、これまで、日本中の献血からの約10,000バッグからなるプール血漿は、4LogIU/mLを超えていなかった。日本の最新の流行期からのほぼ独占的な血漿を含む検査したプール血漿のバッチ以降、日本中のドナーからの全てのプール血漿中のB19V DNA量は将来4LogIU/mLの制限を上回りそうにない。

IV群は低ウイルス量（B19V DNA<4LogIU/mL）の感染の後一末期に相当する。IV群とI群からIII群までの間のCLEIA-B19Vの反応性に明らかな違いがある。IV群サンプルのCLEIA陽性結果は、ウイルス量が8LogIU/mLであった1人のドナーを除いて、非特異性であると見なすことができる。松倉らは、B19V感染がB19Vの症状がない感染した献血者で長期間（約2-4年）続くことを報告した、しかし彼らはIgG抗B19Vの高レベルとCLEIA-B19Vで検出可能でないかもしれない低ウイルス量を維持した。その上、我々はCLEIA-B19V陰性献血の3.1%はIgG抗B19Vが陽性、且つB19Vのウイルス量が低いことを示した。316のCLEIA-B19V陽性およびB19V DNA陰性サンプルは、IV群献血の大部分と類似した、CLEIA-B19V非特異的と考えられている。これらの理由から、率の変動は異なる期間、或いは異なる集団に応じて、IV群の8つの献血(2.5%)は低レベルB19V DNAをもつ献血者であると考えられた（それはまた、CLEIA-B19Vの非特異反応をもつサンプルの間で一定の率で存在しそうだった）。CLEIA-B19V非特異性にもかかわらず、多くのその様なドナーは繰り返し反応した。通常のスクリーニングにおける実際の非特異性（0.03%）がCLEIA-B19V導入前の予備試験での671のB19V DNA陰性サンプルを用いたそれ（0.15%）よりも低かった（未発表のデータ）ため、明らかに、分析で使われるB19V反応性MoAbsは血漿中の幾つかの未知の交差反応抗原をピックアップする可能性がある。

B19Vパネルサンプルの遺伝子型1、2および3aはCLEIA-B19Vにより検出可能であったけれども、遺伝子型1は過去15年間北海道で流行している唯一の株であった。現在、遺伝子型1は世界中で最も一般的な遺伝子型であるが、遺伝子型3も最近米国および多くの他の国で検出されている。札幌市の別の医療機関の遡及調査によれば、104のB19V DNAサンプルが過去28年間に患者から分析され、そして全ての株は遺伝子型1に分離された。B19Vの流行株の2つの強烈な改変は、1980年代後期と1990年代後期に起こった。後者は、本研究でA亜群からB亜群までの変化の段階に対応した。これらの結果は、B19Vの遺伝子型1が過去15年間北海道で流行している支配株の1つであったことを示唆する。2008年だけ散発的に検出したC亜群の2株は北海道で流行している株ではなさそうだった。

一般に、約5～6LogIU/mL未満のB19V DNAを持つIII群とIV群に対応した大部分のサンプルはB19V中和抗体の高力価を含むので、約5LogIU/mLのウイルス量が輸血感染B19V感染症（TT-B19V）に対する感染性閾値レベルと認められ始めていた。Hourfarらは、ドナーのウイルス量が5LogIU/mL以下の16人のレシピエントでB19V DNAが検出されなかった、それとは逆にB19Vウイルス量が5LogIU/mLより大きい血液製剤を輸血された18人のレシピエントの内9人でB19V DNAが確認されたことを報告した。しかしながら、佐竹らは日本でCLEIA-B19Vの導入前に5つの確定TT-B19V症例と3つの可能性があるTT-B19V症例を報告した。1つの確定症例と1つの可能性症例は、約3LogIU/mLのウイルス量の血液に起因していた。TT-B19Vを引き起こした8つの血液成分の内、7つはIII群の型であり、そして1つはII群の型であった。CLEIA-B19Vは約6.3～6.4LogIU/mLを超えているB19V DNA量の血液に起因するTT-B19Vのリスクを低減させることができるにもかかわらず、CLEIA-B19Vスクリーニング感度は輸血用血液によるTT-B19Vを完全に予防するにはまだ不十分である。事実、CLEIA-B19Vの導入後の確定TT-B19Vの最初の症例が日本で最近明らかにされ（未発表のデータ）。従って、CLEIA-B19Vの感度は将来改善されなければならない、もしそうでなければ選択的スクリーニングは溶血性貧血患者、免疫不全患者および妊婦の様なハイリスクレシピエントのために血液製剤で主張しなければならない。代わりに、II群とIII群のサンプルを完全に除去するIgM抗B19Vのための新しい血清学的スクリーニングシステムの組合せは、TT-B19Vの予防のための戦略の1つである場合がある。

B19Vの2つの新しい遺伝子型、遺伝子型2と3は過去15年間北海道の献血者の間で検出されていなかった。しかし、B19Vは主に呼吸器系を介して感染する、そして将来、B19Vの遺伝子型2および3は日本における輸入感染症として潜在的に広がることができた。従って、B19V

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	の全ての既知遺伝子型が CLEIA-B19V によって検出可能である結果が血液の安全性のために大きな重要性を持つ間、継続的なモニタリングは3つの既知遺伝子型の全てを検出するのに必要である。Koppelman らによる提案に従って、更なる製造のための血漿スクリーニング用 B19V NAT は臨床感度が高く、そして全ての変異型を検出することを証明する十分な評価を必要とする。 結論として、CLEIA-B19V はプール血漿中のの B19V のウイルス量だけでなく、輸血用血液の TT-B19V のリスクの低減にも大いに貢献している。更に、本研究は日本の CLEIA-B19V スクリーニングが米国およびヨーロッパの B19V に関係した勧告に対応することができることを示す。TT-B19V の予防のために CLEIA-B19V の感度を強化するための開発が必要であるが、例え B19V の新しい変異型が出現するとしても、血清型が大きく変化しない限り、CLEIA-B19V はまだ B19V の変異型献血をスクリーニングする適切な血清学的方法でありそうだ。	

報告企業の意見	今後の対応
ヒトパルボウイルス B19（Human parvovirus B19：B19）は、パルボウイルス科(Parvoviridae)パルボウイルス亜科(Parvovirinae)エリスロウイルス属(Erythrovirus)に属するエンベロープを持たない極めて小さな（約 20～26nm）1本鎖 DNA ウイルスで、輸血や血漿分画製剤による伝播が報告されている。他のウイルスに比べて、血漿分画製剤の製造工程での不活化・除去が困難であり、本ウイルスの伝播リスクを完全に否定することはできないため、1996 年 11 月より、使用上の注意に B19 についての記載を行い注意喚起を図ってきた。万一、原料血漿に B19 が混入したとしても、Canine parvovirus（CPV）をモデルウイルスとしたウイルスクリアランス試験成績及び B19 を用いた不活化･除去試験の結果から、本剤の製造工程において不活化・除去されると考えている。なお、原料血漿への B19 混入量低減のため、B19 ミニプール NAT が米国の原料供給元で行われている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

Impact of chemiluminescent enzyme immunoassay screening for human parvovirus B19 antigen in Japanese blood donors

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BACKGROUND: To reduce the risk of human parvovirus B19 (B19V) transmission through contaminated blood for transfusion and plasma-derived products, the Japanese Red Cross (JRC) Blood Centers introduced B19V antigen screening by chemiluminescent enzyme immunoassay (CLEIA-B19V) in 2008.

STUDY DESIGN AND METHODS: Donor samples that were positive by CLEIA-B19V screening were tested for B19V DNA. The sensitivity of CLEIA-B19V was tested using samples of all three genotypes and B19V DNA–positive donations. B19V DNA–positive donations and pooled plasma were quantitatively assayed for B19V DNA. B19V DNA–positive donations were phylogenetically analyzed by polymerase chain reaction direct sequencing.

RESULTS: The sensitivity of CLEIA-B19V was inferred to be approximately 6.3 log IU/mL with the genotype samples and 6.4 log IU/mL with B19V DNA–positive donor samples. Of 417 CLEIA-B19V–positive samples from 1,035,560 donations in Hokkaido, Japan, 101 were positive for B19V DNA. The 198 strains of B19V DNA–positive donations in Hokkaido over the past 15 years clustered exclusively with Genotype 1. After introduction of CLEIA-B19V, the viral load for B19V DNA in all 772 pooled plasma for fractionation from donors in nationwide Japan did not exceed 4 log IU/mL.

CONCLUSION: CLEIA-B19V can detect all three genotypes of B19V (viral load >6.3 log IU/mL) and limit the viral load (<4 log IU/mL) in pooled plasma, and thus such screening has further reduced the risk of transfusion-transmitted B19V infection. These results show that CLEIA-B19V screening at the JRC Blood Centers can be an alternative approach to comply with recommendations regarding B19V in the United States and Europe.

Human parvovirus B19 (B19V) causes a variety of diseases in humans, such as erythema infectiosum or fifth disease in children, aplastic crisis or chronic pure red blood cell (RBC) aplasia in hemolytic anemia patients, fetal hydrops or fetal death in pregnant women, and persistent anemia in immunocompromised patients.[1] Although the B19V is mainly transmitted via the respiratory route, blood and plasma-derived products, especially clotting factors, contaminated with high levels of B19V DNA from blood donors, with high viral load but without symptoms, have also been shown to be infectious.[2-4] B19V is a nonenveloped viral pathogen and inactivation methods like solvent/detergent treatment are ineffective for reduction of infectious virus in plasma.

ABBREVIATIONS: B19V = human parvovirus B19; CLEIA = chemiluminescent enzyme immunoassay; COI = cutoff index; JRC = Japanese Red Cross; RHA = receptor-mediated hemagglutination assay; TT-B19V = transfusion-transmitted B19V infection; U-PCR = universal real-time polymerase chain reaction.

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To reduce the risk of B19V transmission through contaminated blood and plasma-derived products, the Japanese Red Cross (JRC) Blood Centers implemented a B19V screening with receptor-mediated hemagglutination assay (RHA) in September 1997 (this had already been implemented in April 1996 on a trial basis in Hokkaido, Japan). RHA is an in-house B19V antigen detection method in which glutaraldehyde-fixed RBCs are agglutinated via B19V particles via globoside on the RBC membrane under acidic conditions (pH 5.2-5.8).[5,6] This resulted in a significant reduction in the contamination level of B19V in the plasma pool.[7,8]

More recently, plasma pools for further manufacture into some, but not all, plasma derivatives are required not to exceed 4 log IU/mL for B19V DNA in Europe,[9-11] while in the United States, a similar limit is recommended for all plasma pools.[12] For this reason, manufacturers are screening plasma minipools using a nucleic acid amplification technology (NAT) to detect B19V DNA and to remove units with high viral loads. Although RHA has greatly contributed to the reduction of B19V viral load in plasma pools for plasma derivatives as well as in blood for transfusion, the detection limit of RHA (approx. 10 log IU/mL)[6] is insufficient to ensure that B19V DNA loads in plasma fractionation pools do not exceed 4 log IU/mL. In 2008, to improve assay sensitivity, the JRC Blood Centers introduced B19V antigen screening by chemiluminescent enzyme immunoassay (CLEIA-B19V). CLEIA-B19V is a two-step sandwich assay and mouse monoclonal antibodies (MoAbs) against B19V with different specificities are used.

B19V has been classified into three genotypes, 1, 2, and 3.[13] Genotypes 1 and 2 are found in the United States, Europe, and other Western countries, while Genotype 3 is mostly restricted to sub-Saharan Africa and South America.[14-16] In northern and central Europe, Genotype 2 strains have been frequently found in tissue samples of older individuals. It was reported to be an ancestral virus circulated in humans in this region until the 1970s and then replaced by Genotype 1.[17] In addition to the three main genotypes, other subtypes (1b) of Genotype 1 strains in Vietnamese patients and subtypes (3b) of Genotype 3 strains in Brazil and Europe have been described.[15,18,19] The European Pharmacopoeia and the US Center for Biologics Evaluation and Research in the Food and Drug Administration (FDA) recommended that all three genotypes of B19V should be detected by NAT procedures.[12,20] Some of the currently available commercial kits and in-house B19V DNA assays fail to detect or underquantify the recently identified Genotypes 2 and 3.[21] In these circumstances, no report has been available on a survey for B19V genotyping in Japanese blood donors and it is unclear whether the CLEIA-B19V can be an alternative approach to comply with these recommendations regarding B19V. Therefore, we studied the possibility of detection of all genotypes by CLEIA-B19V and measured the B19V DNA loads in source plasma for fractionation pooled from Japanese donations and surveyed genotypes of B19V among blood donors in Japan's northern island of Hokkaido over the past 15 years.

MATERIALS AND METHODS

Blood donor screening for B19V antigen

At the JRC Blood Centers, all blood donations are screened by serologic and NAT tests.[22] Seropositive samples are excluded from the 20-pool-screening triple NAT to reduce the risk of cross-contamination during NAT (Fig. 1). Since 2008, all individual donations have been screened for B19V antigen using a CLEIA-B19V on the CL4800 testing system (Fujirebio, Inc., Tokyo, Japan). CLEIA-B19V is a two-step sandwich assay and mouse MoAbs against B19V with different specificities are used. B19V is sandwiched between two groups of antibodies: one is bound to magnetic particles and another is labeled with alkaline phosphatase (ALP). The amount of B19V antigen is measured by the chemiluminescence of 3(-2′-Spiroadamantane)-4-methoxy-4(-3″-phosphoryloxy)-phenyl-1,2-dioxetane

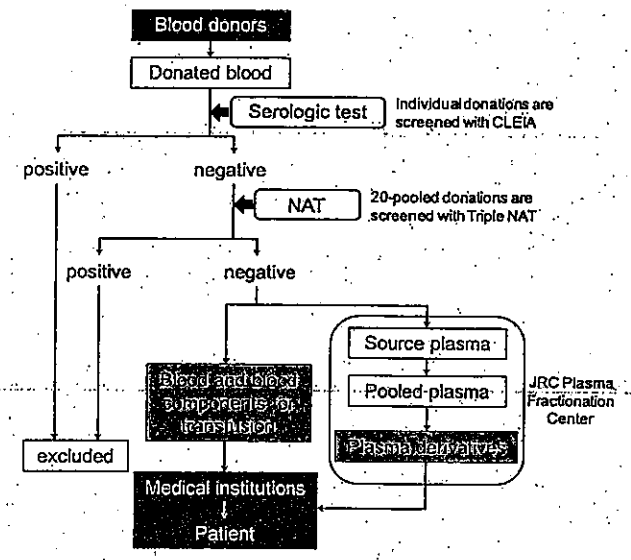


Fig. 1. Flow from blood collection (from donors) to transfusion (to patients) and blood screening tests by serology in single donation format and NAT in minipool format for infectious diseases at the JRC Blood Centers. All blood donations are screened by serologic tests for hepatitis B virus (HBV) surface antigen, B19V antigen, and antibodies against hepatitis B core antigen, hepatitis C virus (HCV), human immunodeficiency virus (HIV) Types 1 and 2, human T-lymphotropic virus Type I, and *Treponema pallidum*.[22] Seronegative samples are subsequently tested by the 20-pool-screening NAT for HBV DNA, HCV RNA, and HIV RNA. Seropositive samples are excluded from the triple NAT described above to reduce the risk of cross-contamination.

hydrolyzed by ALP. Samples with a CLEIA-B19V value (cutoff index [COI]) of greater than or equal to 1.0 are deemed to be positive for B19V antigen. Repeat-reactive samples are excluded from blood and blood components for transfusion and for plasma for further manufacturing by the JRC. The CLEIA-B19V–positive samples in Hokkaido are assayed for B19V DNA by a universal real-time polymerase chain reaction (U-PCR) for B19V for further research, as described below.

A total of 682 randomly selected CLEIA-B19V–negative samples from August 2008 to May 2009, from Hokkaido donors were also assayed for B19V DNA by U-PCR for B19V.

U-PCR for B19V and antibody detection

Total nucleic acids were extracted from 0.2-mL plasma samples by using a virus spin kit (QIAamp MinElute, Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. U-PCR for B19V with some modifications was performed using 2× universal probe master (FastStart, Rox; Roche Diagnostics Co., Ltd, Tokyo, Japan) on a real-time PCR system (Model 7500, Applied Biosystems Japan Ltd, Tokyo, Japan) according to the previous reports.[23,24] Briefly, the primer set (forward primer 5′-AATGC AGATGCCCTCCAC-3′, reverse primer 5′-ATGATTCT CCTGAACTGGTCC-3′) and a TaqMan MGB probe 5′ (FAM)-AACCCCGCGCTCTAGTAC-(MGB) 3′ were selected from the most conserved sequences and amplified a 193-bp fragment of the nonstructural protein (NS1) gene of B19V. Amplification reactions were initially heated to 95°C for 10 minutes and then subjected to 50 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C for 1 minute. The sensitivity of the U-PCR for B19V was evaluated preliminarily with two diluted samples (viral load of approx. 5 log IU/mL) from B19V Genotype 3a panels (M1655, from maternal plasma; C2005, from cord blood) provided by Prof. J.-P. Allain (University of Cambridge, Cambridge, UK).[25] The analytical sensitivities of U-PCR for Genotypes 1, 2, and 3a of B19V were determined to be 15.2 IU/mL (95% confidence interval [CI], 7.8-83.9 IU/mL), 9.9 IU/mL (95% CI, 5.7-36.9 IU/mL), and 16.5 IU/mL (95% CI, 8.6-81.4 IU/mL), respectively, by probit analysis using the World Health Organization (WHO) International Genotype Panel for B19V (NIBSC 09/110; Center for Biologics Evaluation and Research Parvovirus B19 Genotype Panel 1), which comprises three different members, that is, Member 1, Member 2, and Member 3 (M1-M3); these represent Genotypes 1, 2, and 3a of B19V, containing 5.98, 5.94, and 5.97 log IU/mL B19V DNA, respectively, based on the data returned in the collaborative study by 27 laboratories when using quantitative assays for B19V DNA,[26] while it is the policy of the WHO not to assign values to members of NAT panels. Quantitative assays were performed with six standard samples prepared by 10-fold serial dilution of a B19V-positive plasma (2.7, 3.7, 4.7, 5.7, 6.7, and 7.7 log IU/mL, Genotype 1), which were calibrated using a commercially available quantification kit (LightCycler Parvovirus B19V quantification kit, Roche). U-PCR–positive specimens were assayed for immunoglobulin (Ig)M and IgG anti-B19V by enzyme-linked immunosorbent assays (parvo-IgM and parvo-IgG, Denka Seiken, Tokyo, Japan) according to the manufacturer's protocol.

Genotypic specificity of CLEIA-B19V

Since B19V genotype panels of higher B19V viral load were needed to evaluate the genotypic specificity of CLEIA-B19V, some intermediate virus stocks of approximately 10^8 IU/mL, which were used for formulating the First WHO International Reference Panel for parvovirus B19 genotypes,[26] were quantitatively evaluated by U-PCR for B19V as described; viral loads were 8.28, 8.82, and 8.48 log IU/mL, for Genotypes 1, 2, and 3a (MS1-MS3), respectively. Each sample was 10-fold serially diluted at three different concentrations and tested in three assay runs by CLEIA-B19V.

B19V sequence analysis

A total of 105 randomly selected B19V DNA–positive samples from April 1996 to January 2008 (by RHA screening) and 93 B19V DNA–positive samples from February 2008 to September 2011 (by CLEIA-B19V screening) from donors in Hokkaido were phylogenetically analyzed by direct sequencing of PCR products. The period included two recent outbreaks of erythema infectiosum in the first half of 2007 and of 2011.[27] Viral DNA fragments for genotyping were obtained by PCR amplification of a 724-bp region of the NS1/VP1u junction in the B19V genome with conserved primers (forward primer 5′-GGACCAGTTCA GGAGAATCAT-3′, reverse primer 5′-CCAGGCTTGTGTAA GTCTTC-3′). PCR for B19V was performed using a DNA polymerase (TaKaRa ExTaq HS, Takara Bio, Inc., Tokyo, Japan) according to the manufacturer's protocol on a PCR system (GeneAmp 9700, Applied Biosystems). The amplification products were sequenced on both strands with a cycle sequencing kit (BigDye Terminator, Version 3.1, Applied Biosystems) and a genetic analyzer (ABI 3100 or 3700, Applied Biosystems). The sequences were analyzed with computer software (GENETYX-Win, Version 9.11, Software Development, Tokyo, Japan) and aligned together with reported B19V strains with a computer program (CLUSTAL W, Version 1.83).[28] A phylogenetic tree was constructed based on the various sequences of NS1/VP1u region (1709-2392 nt in AF162273) by the neighbor-joining method and the final tree was obtained by a computer program (MEGA, Version 5.05). Bootstrap values were determined by resampling 1000 of the data sets.

The nucleotide sequence data reported in this article are available in the DDBJ/EMBL/GenBank nucleotide

sequence databases with the Accession Numbers AB691331 to AB691528 for HP001 to HP198, respectively.

Contamination levels of B19V in plasma pools

B19V DNA in pooled plasma for fractionation in the JRC Plasma Fractionation Center from May 2003 to February 2012 was isolated from 0.2-mL plasma samples by using an automated nucleic acid isolation system (MagNA Pure LC system, Roche) and the B19V DNA loads were measured by using a B19V quantification kit (LightCycler, Roche) according to the manufacturer's instructions. Each batch of pooled plasma consisted of approximately 5000 to 10,000 bags of source plasma stored for over 6 months (inventory hold) from nonremunerated voluntary donors from 2003 to June 2011 throughout Japan. Of a total of 3072 batches, 772 consisted of plasma screened only by CLEIA-B19V.

Statistical analysis

The sensitivity and 95% CI of the U-PCR test, standard deviation (SD), and regression line were calculated with computer software (Excel 2007, Microsoft Corp., Redmond, WA).

RESULTS

Blood donor screening by CLEIA-B19V in Hokkaido

Of 1,035,560 blood donations from Hokkaido screened by the CLEIA-B19V between February 2008 and September 2011 (44 months), 417 (0.04%) were repeatedly positive, including 101 (0.01%) U-PCR–positive samples (Fig. 2). Of 101 B19V DNA–positive donations, 67 (66%) were detected from December 2010 through June 2011 during the epidemic season (Fig. 2). The 316 (0.03%) CLEIA-B19V–positive, B19V DNA–negative donations came from 171 donors, including 61 repeatedly positive donors.

Donor samples, positive by both B19V DNA and CLEIA-B19V (n = 101), were divided into four phases of infection (provisionally named Groups I, II, III, and IV) according to the presence of anti-B19V: Group I (the early phase of infection)—negative for IgM and IgG anti-B19V; Group II (the early to middle phase of infection)—IgM positive and IgG negative; Group III (the middle to late phase of infection)—IgM and IgG positive; and Group IV (the late to terminal phase of infection)—IgM negative, IgG positive. One of the Group IV donors showed a high level of B19V DNA (>8 log IU/mL; Table 1). This donor can be regarded as being in the transition period from Group III to Group IV (not terminal phase). Eight donations of Group IV (viral load < 4 log IU/mL) came from five donors, one of whom gave positive donations on four occasions.

As shown in Table 1, the Group I and II donations (n = 63) showed high B19V DNA loads of greater than 6 log IU/mL, whereas Group III donations (n = 29) that were positive for B19V-IgG, in addition to B19V-IgM, showed viral loads lower than Group I and II donations. Most of Group IV donations exhibited low viral loads (<4 log IU/mL).

Of 682 samples that were negative by CLEIA-B19V, 21 (3.1%) were positive for IgG anti-B19V and low viral loads of B19V (<4 log IU/mL).

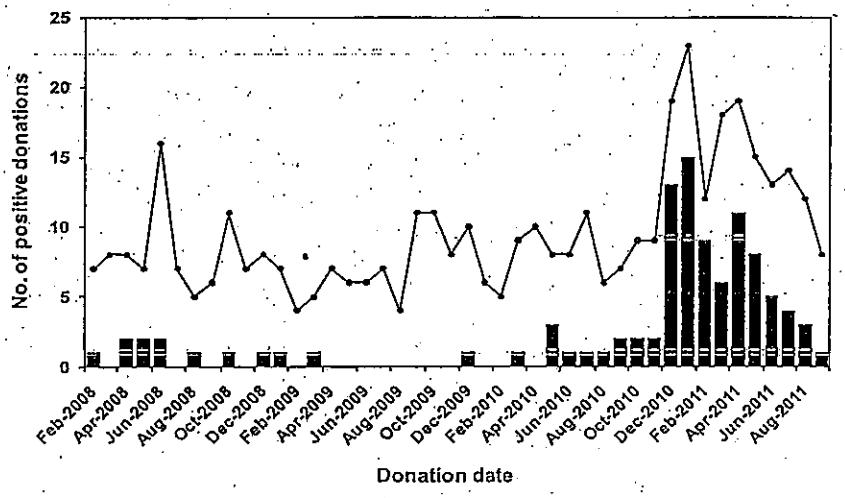


Fig. 2. B19V screening in blood donations by CLEIA-B19V from February 2008 to September 2011 in Hokkaido, Japan. Among 1,035,560 blood donations, 417 (0.04%) were repeatedly positive by CLEIA-B19V (—•—), including 101 (0.01%) U-PCR–positive samples. Of 101 B19V DNA–positive donors (▬), 67 were detected from December 2010 through June 2011 during the most recent epidemic season in Hokkaido.

TABLE 1. B19V DNA loads and immunoglobulin class of B19V antibodies in B19V DNA–positive donations in Hokkaido

B19V DNA load (log IU/mL)	Number of B19V DNA–positive donations	Number of Group I*	Number of Group II*	Number of Group III*	Number of Group IV*
>12	20	19	1	0	0
11 to <12	19	13	6	0	0
10 to <11	7	5	0	2	0
9 to <10	6	6	0	0	0
8 to <9	8	4	0	3	1
7 to <8	18	7	1	10	0
6 to <7	14	1	0	13	0
5 to <6	1	0	0	1	0
4 to <5	0	0	0	0	0
3 to <4	1	0	0	0	1
2 to <3	2	0	0	0	2
<2	5	0	0	0	5
Total (%)	101	55 (54.5)	8 (7.9)	29 (28.7)	9† (8.9)
Mean ±SD (range) B19V DNA load (log IU/mL)	9.0 ± 2.9 (1.5-12.3)	10.6 ± 1.8 (6.5-12.3)	11.1 ± 1.4 (7.6-12.1)	7.3 ± 1.1 (5.6-10.3)	2.8 ± 2.2 (1.5-8.4)

* Group I, B19V-IgM and IgG negative; Group II, B19V-IgM positive and IgG negative; Group III, B19V-IgM and IgG positive; Group IV, B19V-IgM negative and IgG positive.
† The eight donations (viral load < 4 log IU/mL) came from five donors, one of whom gave positive donations on four occasions.

Correlation of CLEIA-B19V value and B19V DNA load in genotype panels and donor samples

Figure 3A shows the CLEIA-B19V values (the mean of three measurements) corresponding to B19V DNA loads of each genotype panel member (MS1-MS3). A highly linear correlation was found between the CLEIA-B19V value and B19V DNA load by power approximation ($R^2 > 0.99$). The regression line of the respective genotype panel samples converged in a certain range. The sensitivity of CLEIA-B19V was inferred to be approximately 6.3 log IU/mL (Genotype 1, 6.23 log IU/mL; Genotype 2, 6.11 log IU/mL; and Genotype 3a, 6.23 log IU/mL), based on the B19V DNA load that each regression line intersect with the cutoff value of CLEIA-B19V (1.0 COI).

Figure 3B shows the CLEIA-B19V values corresponding to B19V DNA loads of B19V DNA-positive samples. The maximum value of CLEIA-B19V is set at a COI of 2000. A regression line using 53 samples, with the exception of eight donations of Group IV (viral load < 4 log IU/mL) and 40 donations with CLEIA value of 2000 COI, showed a linear correlation ($R^2 = 0.87$) and almost the same sensitivity (approx. 6.4 log IU/mL) as using the panels of the three genotypes.

Phylogenetic analysis of B19V among blood donors in Hokkaido

The DNA sequences of 198 B19V DNA–positive donations in Hokkaido segregated into Genotype 1 without exception (Fig. 4). The 198 strains of Genotype 1 were divided into at least three minor subgroups, provisionally named subgroups A, B, and C. Subgroup B was consistently detected between 1996 and 2011, whereas Subgroup A disappeared early during this time and Subgroup C emerged only in 2008 (Fig. 4). Among the three subgroups, no significant difference was observed in age, sex, and the geographical distribution in Hokkaido (data not shown). The nucleotide variation between strains from each subgroup ranged from 0.9% to 3.2%. The variation in each subgroup was less than 1.6%. Each major strain from donors during two recent outbreaks of erythema infectiosum in 2007 and 2011 appeared in two different clusters, but all were segregated into Subgroup B and showed high nucleotide similarities (>98.4%, Fig. 4).

Contamination levels of B19V in plasma pools

Table 2 shows the amounts of B19V DNA in the batch of source plasma pooled from donors in Japan nationwide. Of 2118 batches of source plasma consisting of plasma screened by RHA, 365 (17.2%) batches were contaminated with more than 4 log IU/mL B19V DNA. By contrast, with the start of the plasma screening by CLEIA-B19V, B19V contamination in pooled plasma was decreased. After completely switching to CLEIA-B19V screening, the level of B19V DNA load in all 772 plasma pools (that included almost all plasma obtained from the latest epidemic season) did not exceed 4 log IU/mL, which was the level of recommendation in the United States and Europe.

DISCUSSION

We have shown that the sensitivity of CLEIA-B19V is inferred to be approximately 6.3 log IU/mL, when using some intermediate viral stocks previously evaluated by NAT for formulating the First WHO international B19V

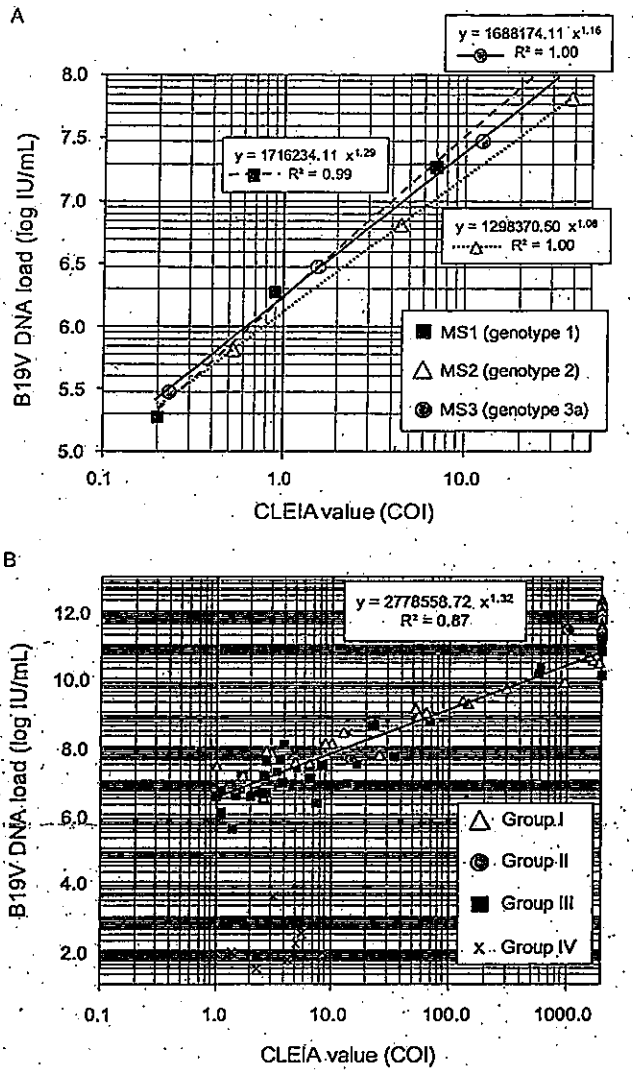


Fig. 3. Correlation of CLEIA value for B19V antigen and B19V DNA load. Samples with a CLEIA value (COI) of greater than or equal to 1.0 are deemed to be positive for B19V antigen. (A) MS1, MS2, and MS3 represent the intermediate (approx. 10^8) viral stocks[26] for Genotypes 1, 2, and 3a, respectively. The B19V DNA load of each genotype viral stock was determined by U-PCR for B19V using B19V DNA standards from B19V-positive plasma (Genotype 1), and each panel was 10-fold serially diluted into three concentrations and testing performed in three assay runs by CLEIA-B19V. (B) A total of 101 B19V DNA–positive donations in Hokkaido from February 2008 to September 2011 were detected by CLEIA-B19V and divided into four phases of infection, provisionally named Groups I, II, III, and IV (see Table 1). The regression line was drawn using only 53 samples except for eight donations of Group IV (viral load <4 log IU/mL) and 40 donations with CLEIA value of 2000 COI of the full-scale range.

genotype panel (Genotypes 1, 2, and 3a)[26] and 6.4 log IU/mL with B19V DNA–positive donor samples. The FDA recently recommended that manufacturers of plasma-derived products implement the following procedures to detect the presence of B19V DNA: 1) B19V NAT for all plasma-derived products should detect all known genotypes of the virus. 2) The viral load of B19V DNA in the manufacturing pools does not exceed 4 log IU/mL.[12] In 2008, serologic B19V screening at the JRC was switched from the conventional agglutination method (RHA) to the CLEIA method to improve sensitivity. Since source plasma for plasma derivatives consists of plasma from nonremunerated voluntary donors in Japan, screening by the CLEIA method also contributes to the safety of all plasma derivatives (Fig. 1). However, it was not clear whether the CLEIA-B19V screening at the JRC Blood Centers can be an alternative approach to comply with the two recommendations of the FDA.

With regard to the first recommendation by the FDA, all three genotypes of B19V have been reported to constitute a single serotype, while these three virus types differ by approximately 10% to 15% over the entire genome.[29] Since CLEIA-B19V is a detection system based on antigen-antibody reaction, it is reasonable to detect all three genotypes of B19V with almost equal sensitivity. For the second recommendation by the FDA, we measured the B19V DNA loads in source plasma for fractionation pooled from donors in Japan nationwide. CLEIA-B19V screening, and subsequent exclusion of B19V-positive units, has markedly reduced the viral load (<4 log IU/mL) in the pooled plasma. Therefore, the CLEIA-B19V is found to comply with two recommendations of the FDA as a screening method.

We provisionally divided B19V DNA–positive donations detected by CLEIA-B19V screening in Hokkaido into the four groups based on the phases of infection. Group I and II donations corresponded to early-middle phase of B19V infection that are highly viremic and are considered to be reliably detected by CLEIA-B19V. B19V-positive plasma with high viral load has a greater risk of cross-contamination on pooled NAT screening, especially during epidemic season. If B19V NAT screening is introduced, CLEIA-B19V screening has the advantage of being able to exclude B19V-positive plasma with high viral load such as the Group I and II donations before pooled NAT screening. Highly viremic B19V-positive blood donations (Groups I-III) were detected in 93 (0.009%) blood donations in Hokkaido from February 2008 to September 2011. In a recent report from the Netherlands,[30] the prevalence of B19V with high titer (>6 log IU/mL) was 0.006% (411/6.5 million) of all Dutch blood donations in the years 2003 to 2009. Likewise, from the United States, the prevalence of B19V with high titer (>6 log IU/mL) was 0.016% (2/12,529) from 2000 to 2003.[31] These recent incidences, using large blood donations, are very similar to our data from Hok-

TABLE 2. Contamination of B19V DNA in source plasma for fractionation pooled from donors throughout Japan

B19 DNA load (log IU/mL)	Number (%) of batches of plasma pools*							
	Total	Screened by RHA		Screened by RHA or CLEIA-B19V		Screened by CLEIA-B19V		
>6	74	74 (3.5)	365 (17.2)	0 (0)	4 (2.2)	0 (0)	0 (0)	
5 to <6	118	118 (5.6)		0 (0)		0 (0)		
4 to <5	177	173 (8.2)		4 (2.2)		0 (0)		
3 to <4	355	334 (15.8)		6 (3.3)		15 (1.9)		
2 to <3	275	232 (11.0)		10 (5.5)		33 (4.3)		
<2†	2073	1187 (56.0)		162 (89.0)		724 (93.8)		
Total	3072	2118		182		772		

* Each batch of source plasma contained pooled plasma consisting of approximately 5000-10,000 plasma bags stored for more than 6 months (inventory hold) from nonremunerated voluntary donors from 2003 to June 2011 in nationwide Japan.
† Less than the limit of detection or quantitation.

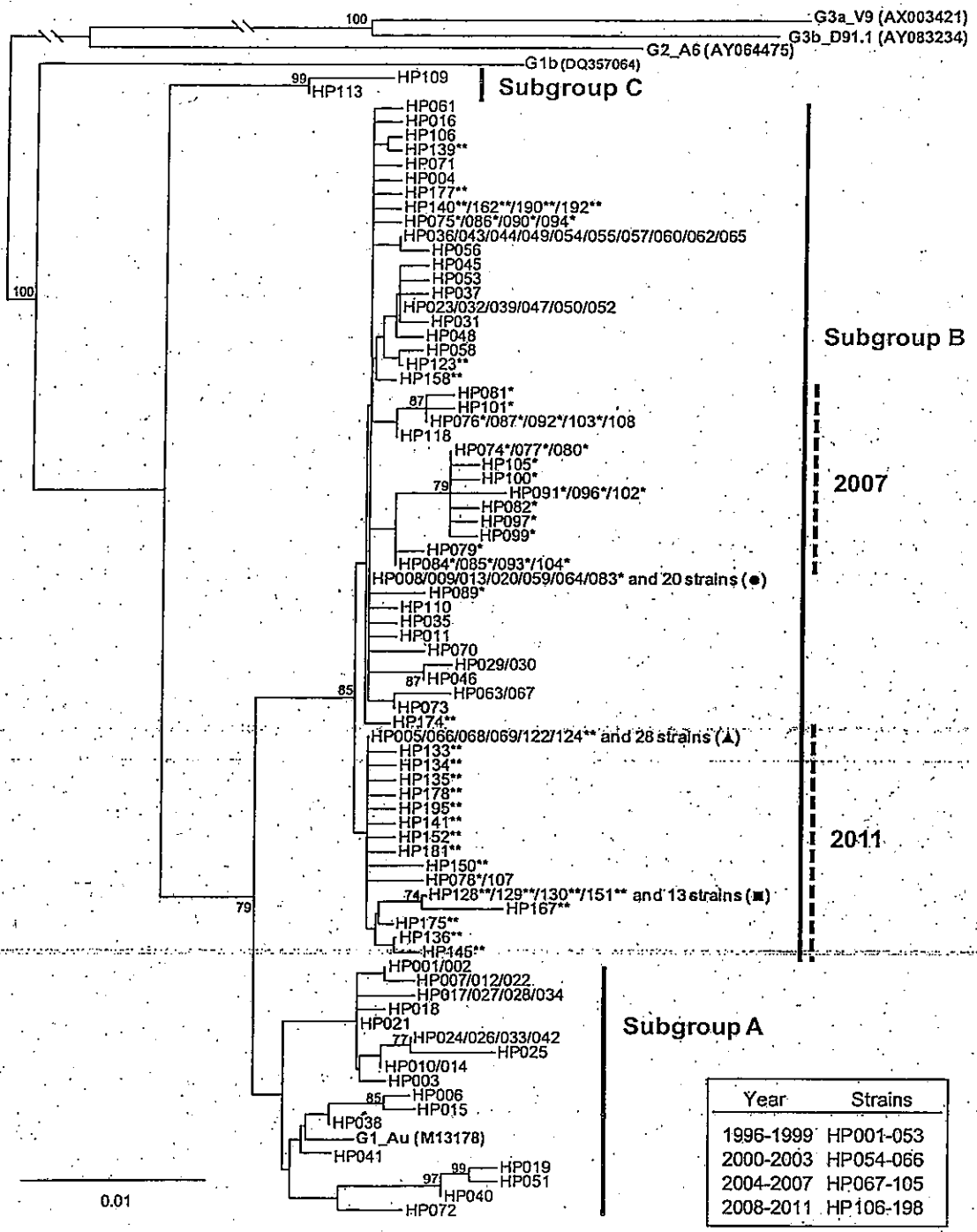
Fig. 4. Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the NS1-VP1u region (684 nt) of B19V among blood donors in Hokkaido (April 1996-September 2011). Genotype 1 is represented by the prototype strain Au (DDBJ/EMBL/GenBank Accession Number M13178). Likewise, Genotype 2, A6 (AY064475); Genotype 3, V9 and D91.1 (AX003421 and AY083234, respectively). Bootstrap values of greater than 70% are indicated for the major nodes as a percentage of the data obtained from 1000 resamplings. All 198 strains (HP001-198) sorted by donation date from blood donors in Hokkaido were segregated into Genotype 1. The 198 strains of Genotype 1 were at least divided into three minor subgroups, provisionally named Subgroups A, B, and C. Major strains from donors during two recent outbreaks of erythema infectiosum in 2007 and 2011 were segregated into Subgroup B: asterisks indicate strains obtained from donors during epidemic season of 2007 (*) and of 2011 (**).

kaido. In Japan, the epidemic cycle for symptomatic B19V infection, which correlates with an increase of B19V DNA–positive donors, has been reported to occur at intervals of 4 to 6 years, as in the Netherlands.[27,30] During the B19V epidemic season in Hokkaido, many B19V strains were found to share 100% nucleotide identity. Despite almost the same epidemic intervals between Hokkaido and nationwide in Japan, it is of interest to discover whether a similar trend will be observed in other parts of Japan.

In contrast to Groups I and II, the antigen detection by CLEIA-B19V may be unstable in the presence of excess of antibodies in the Group III samples. In fact, there was a considerable variation in the CLEIA-B19V values, especially in donations with B19V DNA around 7 log IU/mL. Thus, not all donations with such a level of DNA around 7 log IU/mL may be detected by CLEIA-B19V, although the sensitivity of CLEIA-B19V is inferred to be approximately 6.4 log IU/mL. So far, however, in plasma pools consisting of approximately 10,000 bags from donations throughout Japan, there were none exceeding the limit (4 log IU/mL). Since batches of pooled plasma tested contain almost exclusively plasma from the latest epidemic season in Japan, the B19V DNA load in all the plasma pools from donors throughout Japan is unlikely to exceed the 4 log IU/mL limit in the future.

Group IV corresponds to the late-terminal phase of infection with low viral loads (B19V DNA < 4 log IU/mL). There is an apparent difference in the reactivity of CLEIA-B19V between Group IV and Groups I to III. CLEIA-positive results in Group IV samples can be regarded as likely to be nonspecific, with the exception of one donor whose viral load was 8 log IU/mL. Matsukura and coworkers[32] reported that B19V infection continued over a long period (approx. 2-4 years) in infected blood donors without symptoms of B19V, although they retained high levels of IgG anti-B19V and low viral load that may not be detectable with CLEIA-B19V. Moreover, we showed that 3.1% of CLEIA-B19V–negative blood donations were positive for IgG anti-B19V and low viral load of B19V. The 316 CLEIA-B19V–positive and B19V DNA–negative samples are regarded as CLEIA-B19V nonspecific, similar to most of the Group IV donations. For these reasons, eight (2.5%) donations of Group IV were considered to be the blood donors with low levels of B19V DNA, which were likely to also exist at a constant rate among samples with nonspecific reaction of CLEIA-B19V, while the rate varies depending on the different periods or populations. Despite CLEIA-B19V nonspecificity, many such donors are repeatedly reactive. Apparently, B19V-reactive MoAbs used in the assay may pick up some unknown cross-reactive antigens in plasma, while the actual nonspecificity (0.03%) in routine screening is lower than that (0.15%) with the use of 671 B19V DNA–negative samples in preliminary studies before the introduction of CLEIA-B19V (unpublished data).

Although Genotypes 1, 2, and 3a of B19V panel samples are detectable by the CLEIA-B19V, Genotype 1 was the only strain circulating in Hokkaido over the past 15 years. At the moment, Genotype 1 is the most common genotype in the world, but Genotype 3 has recently been



Year	Strains
1996-1999	HP001-053
2000-2003	HP054-066
2004-2007	HP067-105
2008-2011	HP106-198

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also detected in the United States[33] and many other countries.[19] According to the retrospective study[34] of another medical institution in Sapporo, Hokkaido, 104 B19V DNA samples were analyzed from the patients during the past 28 years, and all strains were segregated into Genotype 1. The two drastic alterations of endemic strains of B19V occurred in the late 1980s and the late 1990s. The latter corresponded to a period of change from Subgroup A to Subgroup B in the present study. These results suggest that Genotype 1 of B19V was one of the dominant strains circulating in Hokkaido over the past 15 years. Two strains in Subgroup C, sporadically detected only in 2008, were unlikely to be strains circulating in Hokkaido.

Because most samples corresponding to Groups III and IV, with B19V DNA less than approximately 5 to 6 log IU/mL, in general, contain a high titer of B19V-neutralizing antibodies, a viral load of approximately 5 log IU/mL was starting to become accepted as an infectious threshold level for transfusion-transmitted B19V infection (TT-B19V).[31,35,36] Hourfar and colleagues[36] reported that B19V DNA was not detected in 16 recipients, where the viral load in the donors was below 5 log IU/mL, whereas B19V DNA was confirmed in 9 of 18 recipients who were transfused with blood products with B19V viral load greater than 5 log IU/mL. However, Satake and colleagues[37] reported five established and three probable TT-B19V cases before introduction of CLEIA-B19V in Japan. One established and one probable case were caused by blood with viral load of approximately 3 log IU/mL. Of eight blood components that caused TT-B19V, seven were the type of Group III and one was of Group II. Although CLEIA-B19V can reduce the risk of TT-B19V caused by blood with B19V DNA load exceeding approximately 6.3 to 6.4 log IU/mL, the sensitivity in CLEIA-B19V screening is still insufficient to completely prevent TT-B19V by blood for transfusion. In fact, the first case of the established TT-B19V after introduction of CLEIA-B19V was recently determined in Japan (unpublished data). Therefore, the sensitivity of CLEIA-B19V should be improved in the future, otherwise selective screening should be advocated in blood products for high-risk recipients such as hemolytic anemia patients, immunocompromised patients, and pregnant women. Alternatively, a combination of a new serologic screening system for IgM anti-B19V to completely eliminate the samples of Groups II and III may be one of the strategies for prevention of TT-B19V.

The two new genotypes of B19V, Genotypes 2 and 3, have not been detected among blood donors in Hokkaido over the past 15 years. However, B19V is mainly transmitted via the respiratory route, and in the future, Genotypes 2 and 3 B19V could potentially spread as an imported infectious disease in Japan. Therefore, continuous monitoring is necessary to detect all three known genotypes, while the result that all known genotypes of B19V are detectable by CLEIA-B19V has great significance for blood safety. According to the proposal by Koppelman and colleagues,[38] B19V NAT for screening of plasma for further manufacture requires thorough evaluations to prove that the clinical sensitivity is high and all variants are detected.

In conclusion, CLEIA-B19V has greatly contributed in the reduction not only of the viral load of B19V in pooled plasma, but also the risk of TT-B19V in blood for transfusion. Moreover, this study shows that CLEIA-B19V screening in Japan can comply with recommendations regarding B19V in the United States and Europe. Although a development to enhance sensitivity in CLEIA-B19V for prevention of TT-B19V is needed, even if the novel variants of B19V emerge, as long as the antigenicity has not greatly changed, CLEIA-B19V is still likely to be an appropriate serologic method for blood screening with donations having variants of B19V.

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CONFLICT OF INTEREST

The authors have no conflict of interest for this article.

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研究報告の概要

背景：ヒトパルボウイルス 4（PARV4）は、パルボウイルス B19（B19V）の様に、血漿分画製剤の製造に使用されるプール血漿の汚染物質になりうる新規のパルボウイルスである。B19V の不活性化研究は、B19V は動物のパルボウイルスよりもウイルス不活性化戦略に対してより感受性が高いことが示されている。しかし、PARV4 の不活化は、まだ具体的に研究されていない。

研究デザインと方法：熱、或いは低 pH 条件によるパルボウイルスの処理は、ウイルスゲノムの外在化（カプシドの外に DNA が出ること）の原因となる。ヌクレアーゼ処理を組み合わせたリアルタイム PCR を用いて、外在化ウイルス DNA をアルブミンの液状加熱や低 pH 処理による PARV4、B19V およびマウスの微小ウイルス（MVM）の不活化の間接的な証明として使用した。感染性の研究は B19V と MVM で同時に行った。

結果：PARV4 は B19V よりも液状加熱および低 pH 処理で抵抗性を示したが、PARV4 は MVM ほど抵抗性がなかった。液状加熱および低 pH 処理後のカプシド形成 PARV4 DNA は 2～3log の低減であった。対照的に、MVM がこれらの条件下で安定であり、B19V を効果的に不活化させた。2 価陽イオンは PARV4 カプシドに対する安定化効果を有することが分かった。2 価陽イオンの不存在下では、中性 pH でも PARV4 の力価は低減され、B19V や MVM では観察されていない効果があった。

結論：加熱処理と低 pH インキュベーションの場合、B19V および MVM と比較した時、PARV4 は中間的な抵抗性を示す。2 価陽イオンは PARV4 ウイルスを安定化するために重要でありそうだ。

使用上の注意記載状況・その他参考事項等

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

報告企業の意見	今後の対応
ヒトパルボウイルス4（Human parvovirus 4：PARV4）は、ヒトパルボウイルスB19の近縁ウイルスで、ゲノムに1本鎖DNAをもつノンエンベロープウイルスであり、大きさは20nm程度と比較的小さい。その病原性は現時点で明らかではないが、血漿分画製剤からの伝播が報告されていることもあり、今後、注意深く追加情報をフォローする必要があると考えている。なお、万一原料血漿にPARV4が混入したとしても、Canine parvovirus（CPV）をモデルウイルスとしたウイルスクリアランス試験成績及びB19を用いた不活化・除去試験の結果から、本剤の製造工程において不活化・除去されると考えている。	PARV4 に関する追加情報の入手に努める。

OTHER VIRUSES

Studies on the inactivation of human parvovirus 4

Sally A. Baylis,[1] Philip W. Tuke,[2] Eiji Miyagawa,[3] and Johannes Blümel[1]

BACKGROUND: Human parvovirus 4 (PARV4) is a novel parvovirus, which like parvovirus B19 (B19V) can be a contaminant of plasma pools used to prepare plasma-derived medicinal products. Inactivation studies of B19V have shown that it is more sensitive to virus inactivation strategies than animal parvoviruses. However, inactivation of PARV4 has not yet been specifically addressed.

STUDY DESIGN AND METHODS: Treatment of parvoviruses by heat or low-pH conditions causes externalization of the virus genome. Using nuclease treatment combined with real-time polymerase chain reaction, the extent of virus DNA externalization was used as an indirect measure of the inactivation of PARV4, B19V, and minute virus of mice (MVM) by pasteurization of albumin and by low-pH treatment. Infectivity studies were performed in parallel for B19V and MVM.

RESULTS: PARV4 showed greater resistance to pasteurization and low-pH treatment than B19V, although PARV4 was not as resistant as MVM. There was a 2- to 3-log reduction of encapsidated PARV4 DNA after pasteurization and low-pH treatment. In contrast, B19V was effectively inactivated while MVM was stable under these conditions. Divalent cations were found to have a stabilizing effect on PARV4 capsids. In the absence of divalent cations, even at neutral pH, there was a reduction of PARV4 titer, an effect not observed for B19V or MVM.

CONCLUSION: In the case of heat treatment and incubation at low pH, PARV4 shows intermediate resistance when compared to B19V and MVM. Divalent cations seem important for stabilizing PARV4 virus particles.

Human parvovirus 4 (PARV4) was first detected in 2005, in plasma from an intravenous drug user (IVDU) with general symptoms of viral disease.[1] Since the discovery of PARV4, related viruses, termed hokoviruses, have been identified in cows, pigs, and wild boar, with further homologs found in sheep and bats; viruses more closely related to PARV4 have also been found in primates.[2-7] While little is understood about the biology of these viruses, they cluster in a distinct group compared with other members of the *Parvoviridae* and a new genus, tentatively named Tetraparvovirus, has been proposed (S. Cotmore, personal communication, 2013). PARV4 demonstrates many of the hallmarks of parvoviruses. Both native and recombinant PARV4 particles have a diameter of 20 to 25 nm, consistent with that determined for other parvoviruses.[8-10] The PARV4 genome contains two main open reading frames and has been shown to encode a nonstructural protein and two capsid proteins.[1,11] Although not based on full-length sequences, analysis of the PARV4 genome indicates that the two ends of the viral DNA are related by inverted terminal repeats containing repeated heptanucleotide sequences in the hairpin stems similar to those found in the inverted terminal repeats of avian parvoviruses.[1,12] PARV4, like human parvovirus B19 (B19V), packages both positive and negative DNA strands into virions.[12]

Using a virus discovery technique based on amplification of nucleic acids that are not freely accessible to

ABBREVIATIONS: B19V = parvovirus B19; IVDU(s) = intravenous drug user(s); MVM = minute virus of mice; PARV4 = human parvovirus 4; qPCR = quantitative polymerase chain reaction.

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nuclease digestion, PARV4 was identified during a study of patients with acute viral infection syndrome, after high-risk behavior for human immunodeficiency virus (HIV).[1] This initial PARV4-infected individual was an IVDU coinfected with hepatitis B virus. Several studies have subsequently found PARV4 in IVDUs in Europe, Asia, and the United States as well as in men who have sex with men and febrile patients.[13-17] Such cases are indicative of blood-borne transmission of PARV4, and the virus has been detected in blood donors, for example, in the United States, South East Asia, and Europe.[16-20] The prevalence of PARV4 in donor populations is not well understood and, for example, studies in France have shown differences in the prevalence of PARV4 DNA in donors, from 0% to 24% which may be due to lack of standardization of detection methods or differences in virus epidemiology.[20,21]

The virus has been detected in plasma pools used in the manufacture of plasma-derived medicinal products, particularly those from the United States and also Asia (S. Baylis and J. Blümel, unpublished observations)[17,22,23] and also in associated clotting factor concentrates, that is, preparations of Factor (F)VIII and F IX.[24,25] Products manufactured in the early 1970s were found to be positive for PARV4, and in general, older concentrates were found to be more frequently contaminated with PARV4.[24] A study of recently manufactured concentrates (FVII and FVIII or von Willebrand factor) commercially available in Germany failed to detect PARV4.[26] The porcine homolog of PARV4, porcine hokovirus, has also been identified in porcine FVIII concentrates.[27]

The consequences for recipients of PARV4-contaminated products are not well understood. However, PARV4 DNA has been detected in persons with hemophilia and there is increased PARV4 seropositivity in persons with hemophilia (HIV and/or hepatitis C infected) treated with nonvirally inactivated FVIII and F IX, whereas nonhemophiliac siblings of these patients were found to be seronegative, despite having close household contact.[8,14] A retrospective study of persons with hemophilia, treated with virally inactivated clotting factor concentrates, has demonstrated infection of patients by analysis of anti-PARV4 immunoglobulin M and viremia, with rash and exacerbation of hepatitis being the most common clinical presentations.[28] The implicated products, administered before 1993, had been treated with solvent/detergent (S/D) or were heat treated. Since PARV4 is a nonenveloped parvovirus, S/D treatment would not be expected to be effective for virus inactivation.

It is now well established that B19V has a different susceptibility to pasteurization and low-pH treatment in contrast to model parvoviruses, such as porcine parvovirus and minute virus of mice (MVM), which have all been used in virus inactivation studies.[29,30] Therefore, we have performed experimental studies to investigate the inactivation of PARV4 and compared this to B19V and MVM. As no permissive cell culture systems for PARV4 are available to investigate virus inactivation, we have developed an in vitro assay to determine the sensitivity of PARV4 to pasteurization (wet heat treatment) and low-pH treatment. The assay is based on the observation that, upon heating or exposure to extreme pH, structural changes in the capsids result in the externalization of the genomes of parvoviruses such as B19V and MVM. Externalized DNA can be digested by nuclease treatment, while encapsidated DNA is protected from digestion. The extent of genome externalization has been shown to correlate with loss of infectivity of B19V and MVM.[29-33]

MATERIALS AND METHODS

Viruses and cells

For PARV4 and B19V, virus stocks were viremic plasma donations. The Genotype 1 PARV4 strain has been previously described.[9] The B19V strain, termed DRK1 (Genotype 1 B19V), was provided by W.K. Roth (German Red Cross, Hessen, Germany). Stocks of the p-strain of MVM (ATCC VR-1346) were prepared in A9 murine fibroblast cells (ECACC 85011426). B19V infectivity studies were performed using the human erythroid cell line KU812Ep6.[34] MVM infectivity assays were performed using A9 cells. Infectivity assays for B19V and MVM were performed as previously described.[30]

Pasteurization

Commercial 5% human serum albumin was heated to 59°C in an Eppendorf 5436 thermoblock (Eppendorf, Hamburg, Germany). The temperature was measured throughout the experiment by monitoring an unspiked albumin sample incubated in parallel using a calibrated thermometer. Volumes of preheated albumin were spiked separately with B19V, PARV4, or MVM. Samples of B19V and MVM were titrated for infectivity using KU812Ep6 cells and A9 cells, respectively. In addition, samples of all three viruses were treated with nuclease and assayed for DNA concentration as described below.

Low-pH treatment

Virus was spiked into phosphate-buffered saline (PBS) and the pH was adjusted with 1 mol/L HCl to the target pH and the sample incubated at 37°C for the specified period of time. Samples were withdrawn and immediately neutralized with 1 mol/L NaOH, buffered with phosphate buffer (100 mmol/L, pH 7.4) and subjected to virus titration using susceptible cells (B19V and MVM). At the specified times, samples of all three viruses were taken and subsequently treated with nuclease and assayed for DNA content as described below.

Controls

Controls for cytotoxicity and interference were performed as previously described.[30] In each virus experiment, the titer of B19V or MVM stock was verified by titration on KU812Ep6 cells or A9 cells, respectively. In parallel to each inactivation experiment, the test matrix was spiked at room temperature (heat inactivation) or at neutral pH (low-pH treatment).

Analysis of virus DNA

Samples of each preparation (200 µL) were treated with 250 units of nuclease (Benzonase, Novagen, Darmstadt, Germany) with $MgCl_2$ at a final concentration of 2 mmol/L and incubated at 37°C for 1 hour. Control samples were incubated in the absence of enzyme. Immediately after incubation, samples were extracted using a DNA Blood Mini Kit (QIAamp, Qiagen GmbH, Hilden, Germany).

Quantification of virus DNA was performed using real-time polymerase chain reaction (PCR); primers and probes have been previously described.[17,35-37] Amplification reactions were performed using the LightCycler FastStart DNA MasterPLUS HybProbe kit (Roche Applied Science, Mannheim, Germany). Primers were used at a concentration of 0.5 µmol/L; probes were used at a concentration of 0.1 or 0.2 µmol/L (for MVM and B19V/PARV4, respectively). All assays were performed using the LightCycler instrument (LightCycler 1.5, Roche Applied Science). The amplification conditions were as follows: 95°C for 15 minutes and then 45 cycles of the following sequential steps: 95°C for 15 seconds and 60°C for 1 minute. Fluorescence data were collected during the combined annealing and extension step and detected in Channel F1. Data analysis was performed using Channels F1 and F2.

Standard curves for real-time PCR were created by dilution of stock viruses in the respective experimental matrix. The DNA titers of the stock viruses were determined by comparison to the signal generated from a known concentration of target DNA. This was obtained by taking amplified DNA, from the respective viruses, which had been purified using a spin procedure (QIAquick Qiagen GmbH). The DNA concentration was determined using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Inc., Wilmington, DE). The respective assays were able to detect at least 1000 copies/mL of virus DNA.

RESULTS

Inactivation kinetics during pasteurization

Pasteurization of plasma protein solutions such as human serum albumin is a well-established method prescribed in various pharmacopoeias and shown to be effective against

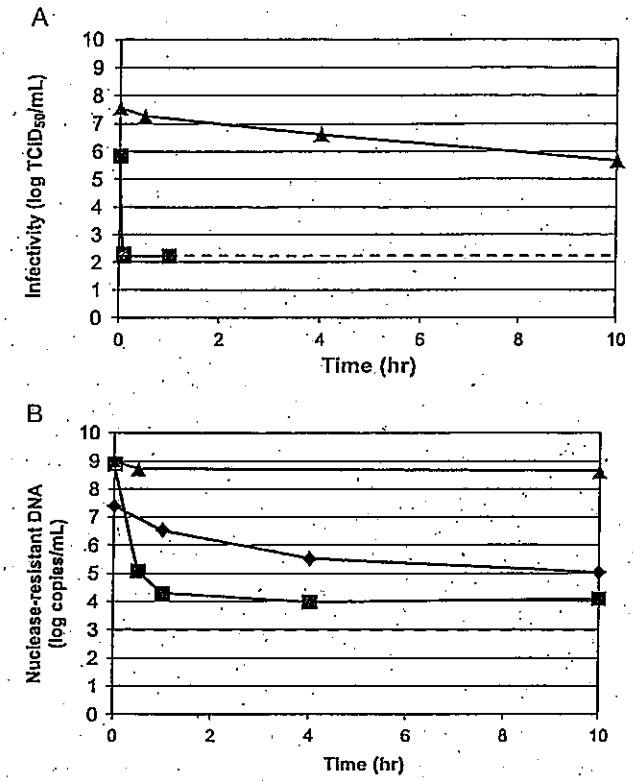

Fig. 1. Inactivation of parvoviruses by heat treatment. (A) Infectivity analysis. Human albumin 5% was heated to 56 or 59°C and spiked 1:20 with B19V or MVM, respectively. Incubation was continued and infectious titer was determined at various time points by titration of B19V on KU812Ep6 cells or A9 cells. The starting point of inactivation kinetics (0 min) represents the infectious titer from a sample spiked at room temperature. (B) DNA encapsidation assay. Human albumin 5% was heated to 59°C and spiked 1:20 with B19V, PARV4, or MVM. Incubation at 59°C was continued and the samples were treated with nuclease and extracted, and DNA titers were determined by qPCR. (◆) PARV4; (■) B19V; (▲) MVM; (- - -) limit of detection.

enveloped viruses. As was expected, immediate inactivation of B19V was observed after inoculation of virus into albumin incubated at 59°C (Fig. 1A). In the case of MVM, after the complete 10-hour incubation period, there was a 1.8-log reduction in infectivity and the inactivation kinetics were very much slower than was observed for B19V. Control samples, diluted in albumin and held at room temperature for the duration of the pasteurization treatment (10 hr) showed no loss of B19V or MVM infectivity.

Investigation of the DNA externalization of B19V and MVM was compared to PARV4 and to the infectivity of B19V and MVM. For B19V, similar to the infectivity experiments, the virus DNA was very rapidly reduced, effective reduction (in the order of 4 log) was observed after 30

TABLE 1. Sensitivity of parvovirus DNA to nuclease after low-pH treatment*

Treatment	Virus (pH)			
	B19V (3.5)	PARV4 (3.5)	PARV4 (4.2)	MVM (3.5)
0 min (– nuclease)	9.1	8.0	7.8	9.6
10 min (+ nuclease)	5.2	6.0	7.3	9.6
2 hr (+ nuclease)	5.3	6.4	7.5	9.5
6 hr (+ nuclease)	4.7	6.3	7.5	9.7
6 hr (+ nuclease), pH 7.0	8.9	6.2	6.7	9.4

* Data are reported as log copies/mL. Parvovirus samples were spiked into the test matrix (PBS without calcium and magnesium, pH 3.5, pH 4.2, or pH 7.0), and incubation continued at 37°C for up to 6 hours. The samples were treated with nuclease and extracted and DNA titers determined by qPCR. The pH of the controls remained stable throughout the incubation period. In parallel experiments, B19V infectivity was immediately inactivated upon pH adjustment, while MVM infectivity was unaffected even after 6 hours of incubation at pH 3.5 at 37°C.

minutes of treatment at 59°C and continued to decrease more slowly over the remainder of the 10-hour incubation period (Fig. 1B). In the case of MVM, much more limited DNA reduction was observed (in the order of 0.5 log) with very slow kinetics over the entire pasteurization period. In the case of MVM, experiments were performed using primers in the NS1 gene as well as the capsid gene. Externalization of parvovirus DNA proceeds in a 3'-to-5' direction, and since the original PCR method targeted the NS1 gene (which is at the 5' end of the genome in parvoviruses), the PCR was repeated using primers at the 3' end to make sure that the relative stability of the MVM DNA was not due to protection of the target region of the genome (i.e., the 5' end) by the virus capsid. The same results were observed whichever PCR method was used for MVM (data not shown). The PCR for B19V targets the NS1 gene, while the PARV4 PCR targets a highly conserved region within the capsid gene. Inactivation of PARV4, as determined by externalization of the virus DNA, proceeded much more slowly than B19V, such that after 1-hour incubation at 59°C, reduction was less than 1 log. After 10 hours, there was a 2.6-log reduction in PARV4 DNA after incubation in albumin at 59°C, far less than was observed for B19V, but greater than MVM.

Inactivation by low-pH treatment and divalent cation dependence for PARV4 stability

Because of the wide use of a variety of plasma product–specific low-pH treatment steps for virus inactivation, the susceptibility of the panel of parvoviruses to low-pH treatment was investigated by spiking viruses into PBS and adjustment of the pH, before incubation at 37°C for up to 6 hours. In the case of B19V, there was an almost immediate loss of infectivity at pH 3.5 upon acidification of the PBS, while there was no loss of MVM infectivity even after prolonged incubation (data not shown). Control samples, diluted in PBS (pH 7.4) and held at 37°C for a period of 6 hours showed no loss of B19V or MVM infectivity. Investigation of the DNA externalization of the panel of viruses indicated that while there was no loss of DNA titer in the case of MVM, with B19V, there was a very rapid decrease as the DNA was externalized and rendered susceptible to nuclease digestion, a pattern that reflected the virus infectivity data (Table 1). No reduction was observed in the hold controls maintained at pH 7.0 over a period of 6 hours for either B19V or MVM. In contrast, it was found that levels of PARV4 DNA declined to a similar extent whether subjected to low pH treatment or maintained at pH 7.0 (Table 1); in some experiments even spiking of PARV4 into buffer solution at neutral pH caused a decrease in titer (data not shown).

In the initial experiments investigating low-pH inactivation (Table 1), Dulbecco's PBS (modified without calcium and magnesium) had been used. Further experiments were performed and it was found that using Dulbecco's PBS[38] containing calcium and magnesium helped to stabilize the PARV4 capsids such that after 2 hours of incubation at pH 3.5 there was no loss of DNA titer compared to a 1.6-log titer in samples incubated in the absence of divalent cations (Tables 1 and 2). This improvement in PARV4 stabilization was observed with both types of divalent cation when PBS was prepared with either calcium or magnesium alone, although Dulbecco's PBS containing calcium and magnesium was more effective. In the case of PBS containing just divalent calcium ions, although some loss of PARV4 DNA (in the order of 0.5 log) was observed at neutral pH over the 6-hour incubation period, there was a greater loss of titer (approx. 1.5 log) under acidic conditions after 6 hours of treatment suggesting that the additional loss in DNA titer was due to the reduction in pH (Table 2); similar results were observed for divalent magnesium cations.

DISCUSSION

In the case of parvoviruses, it was originally believed that pasteurization was ineffective in inactivating this family of nonenveloped viruses. This presumption was based on the use of animal parvoviruses, including MVM and porcine parvovirus, as models to evaluate the efficacy of virus inactivation techniques. Once infectivity assays for B19V were developed, studies demonstrated that this virus was far more sensitive to virus inactivation procedures such as pasteurization and low-pH treatment, used in the production of plasma derivatives, than the model animal viruses.[29,30] It has been well documented that B19V is a frequent contaminant of plasma fractionation pools; however, more recently, PARV4 has also been found to be

TABLE 2. Divalent cations help stabilize PARV4 DNA at low-pH treatment (pH 3.5, at 37°C)*

	Virus/divalent cation(s)				
Treatment	B19V/Ca^{2+}	PARV4/Ca^{2+}	B19V/Mg^{2+}	PARV4/Mg^{2+}	PARV4 Ca^{2+} and Mg^{2+}
0 min (– nuclease)	10.1	8.7	9.9	8.2	8.2
2 hr (+ nuclease)					
pH 3.5	5.3	7.7	5.4	7.3	8.2
pH 7.0	ND	ND	ND	ND	8.4
6 hr (+ nuclease)					
pH 3.5	5.4	7.1	5.9	6.8	ND
pH 7.0	10.3	8.1	10.5	7.3	ND

* Data are reported as log copies/mL. Samples of B19V or PARV4 were spiked into the test matrix (PBS containing calcium, magnesium, or both calcium and magnesium, pH 3.5 or pH 7.0), and incubation continued at 37°C for up to 6 hours. The samples were treated with nuclease and extracted, and DNA titers were determined by qPCR. The calcium and magnesium concentrations were 0.9 and 0.5 mmol/L, respectively.
ND = not determined.

present in some pools and older clotting factor concentrates.[13,17,24] The oldest concentrates in which PARV4 DNA could be readily detected were prepared by simple cryoprecipitation—the earliest lots tested dated to the early 1970s.[24] Nevertheless, PARV4 DNA was also detected in concentrates manufactured by a mixture of precipitation and adsorption and subject to different types of wet and dry heat treatments during the 1980s.[24,25] PARV4 DNA was detected more rarely in concentrates manufactured since the mid-1990s where manufacturing methods were refined and regulatory requirements more stringent to ensure at least one effective step for the reduction of nonenveloped viruses and two for enveloped viruses.[24,25] The probable transmission of PARV4 by older concentrates has subsequently been reported; the implicated concentrates were wet (pasteurization at 60°C) or dry (typically 80°C) heat treated, and a small number were purified by affinity chromatography using specific monoclonal antibodies.[28] A clear role for PARV4 in human disease remains to be defined and contamination of plasma fraction pools cannot be excluded since nucleic acid test screening for PARV4 is not performed; therefore, it is of interest to evaluate the susceptibility of PARV4 to different inactivation strategies used during the manufacture of plasma derivatives.

The tropism of PARV4 is unknown and efforts to culture PARV4 in a variety of cell lines including human fibroblasts and hepatocytes as well as marrow and hematopoietic cells have been unsuccessful (S. Baylis and J. Blümel, unpublished observations; K. Brown, personal communication, 2013). Because there is currently no cell culture system available for PARV4, an in vitro assay was developed whereby inactivation is indirectly measured after treatment of the sample, and a combination of nuclease treatment and quantitative PCR (qPCR) have been used to investigate levels of virus DNA reduction. This assay makes use of a phenomenon that application of heat or low-pH treatment, for example, cause alterations in the capsids of parvovirus particles such that the N-terminus of the VP1 protein become exposed and subsequently the virus DNA is extruded from the intact capsids.[32,33] Using this assay system it was found that PARV4 showed intermediate resistance to pasteurization of albumin when compared to B19V and MVM. In parallel infectivity experiments, B19V was inactivated almost immediately and this observation correlated well with the reduction factors observed for the DNA titers. In the case of MVM, the loss of DNA titer was in the order of 0.5 log after the full 10-hour treatment period; this correlated with a 1.8-log loss of infectivity. It is likely that the initial changes to the VP1 capsid protein, resulting in exposure of the N-terminus of the VP1 protein, may in turn cause the greater observed reduction in infectivity in the case of MVM compared to the reduction in the DNA titer. This phenomenon could be explained by the tighter association of the MVM genome with the virus capsid compared with B19V—where under more extreme conditions, the B19V genome can become completely dissociated from the virus capsid.[32,33] For PARV4, a 2.6-log reduction in DNA titer was observed after the 10-hour incubation. Given the virus loads of up to 7 log copies/mL of PARV4 DNA potentially present in fractionation pools and more than 5 log copies/mL present in, for example, some FVIII concentrates, some residual virus infectivity might be expected, which would be in agreement with the transmission of PARV4 by pasteurized clotting factor concentrates, manufactured in the late 1980s and early 1990s when PARV4-contaminated concentrates were more frequently identified.[24,25,28] The sensitivity of B19V to wet heat treatment can be affected by the composition of the solution and the presence of stabilizers.[39] Similarly it might be expected that stability of PARV4 is affected by the product matrix. In the study by Sharp and colleagues[28,40] in a single case, PARV4 also appeared to have been transmitted by dry heat–treated clotting factors, and again dry heat treatment may not always be so effective for inactivation of B19V. While there is evidence of inactivation of B19V by neutralizing antibodies present in pools with low viral

loads, nothing is known about the levels of anti-PARV4, which might contribute to virus neutralization. Given the frequent identification of PARV4 coinfection with other blood-borne viruses such as HIV and hepatitis C virus, removal of plasma units contaminated with such pathogenic viruses by donor screening may have led to the apparent reduction in PARV4 contamination of plasma fractionation pools.[17]

Investigation of the sensitivity of PARV4 to low-pH treatment identified the importance of divalent cations in the stabilization of PARV4 virus particles. The absence of divalent cations caused instability of PARV4 even at neutral pH; addition of low concentrations of divalent cations helped to stabilize the virus particles. In the case of B19V, reduction of both infectivity and DNA titer occurred very rapidly; in contrast, MVM was completely stable at pH 3.5 at 37°C with no loss of either DNA titer or virus infectivity. Therefore, it might be expected that PARV4 will demonstrate some loss of infectivity under these conditions. Although the pH 3.5 at 37°C represents more stringent conditions than those used for the manufacture of plasma-derived immunoglobulin products, the pH 4.2 data are within the pH range commonly used. Various manufacturing processes are employed with pH values ranging from 4.0 to 4.5, temperatures between 4 and 37°C, and incubation times between 6 hours and 28 days. Incubation at 37°C is usually at pH 4 for not more than 24 hours while incubation at low temperature needs to be extended up to 21 days to achieve effective virus reduction of B19V.[41] Although it is difficult to extrapolate the reduction data from this study to specific products, it seems questionable whether PARV4 is inactivated by such procedures.

Studies of MVM have revealed that the virus can be destabilized by the addition of EDTA, such that incubation at 37°C, at pH 7.2 causes extrusion of much of the viral genome.[33] Divalent cation-binding sites in the MVM particle are critical for genome retention and likely play a role in vivo relating to virus packaging and uncoating. Certain capsid mutations in MVM alter the mechanism of genomic DNA release and are noninfectious at 37°C, but infectious at 32°C.[42] Some parvoviruses, including PARV4, encode an additional protein—small alternatively translated protein, overlapping the amino-terminal portion of the VP2 ORF.[43] In the case of porcine parvovirus, small alternatively translated protein has been shown to colocalize with calreticulin, which acts as a calcium store and is located in the membranes of the endoplasmic reticulum.[44] Until a suitable cell culture system becomes available for PARV4, similar studies will not be possible; nevertheless, divalent cations clearly play an important role in the structural integrity of virus particles. It will be important to define the tropism of PARV4 in the future and to evaluate different cell culture systems for the propagation of this virus to better evaluate virus reduction strategies whether it is for the manufacture of plasma derivatives or for blood components. The implication for recipients of PARV4-contaminated products is still uncertain, particularly since the role of PARV4 in human disease has not been well defined. Recent studies have detected PARV4 in a small number of individuals with encephalitis and also in fetal hydrops, but a definite association with a specific pathology has yet to been demonstrated.[45-47]

In summary, the results from this study demonstrate that PARV4, unlike B19V, is more resistant to virus inactivation strategies used during the manufacture of plasma derivatives.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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別紙様式第２－１

医薬品　研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
一般的名称	—		研究報告の公表状況	http://www.ansm.sante.fr/S-informer/Points-d-information-Points-d-information/Risque-de-transmission-par-le-sang-de-la-maladie-de-Creutzfeldt-Jakob-evolution-de-l-analyse-de-risque-depuis-15-ans-Point-d-Information	公表国 仏国	
販売名（企業名）	—					

研究報告の概要

1990 年から 2010 年までの血液及び血液製剤によるクロイツフェルト・ヤコブ病の感染リスクの分析の変遷を追跡した。
血液及び血液製剤（特に、凝固因子又は免疫グロブリンなどの血漿製剤）によるクロイツフェルト・ヤコブ病（ウシ海綿状脳症（BSE）の原因となる感染性因子）の感染リスクは、1980 年代初めから保健当局の関心の中心であり、英国における「狂牛病」の発生、さらに 1996 年の変異型の最初の患者の発生により 1990 年代に再度関心が高まった。
1990 年から 2010 年までの間にフランス当局がどのようにリスク分析を行ってきたかを、科学的知識の発展に伴って使用、修正された作業仮説とともに説明した。現在、フランスでは 27 例の vCJD 症例が同定されており、最新の予測では、フランスにおいて 60 年間で合計 100 例程度と予想されている。
輸血用血液製剤については、感染リスクは知られているが、このリスクは輸血により得られるベネフィットで相殺される。
反対に、1990 年代からフランスで採用されている製造法で作られた血漿分画製剤では現在まで感染例は記録されておらず、このことは、2000 年代から実施され、血漿分画製剤のリスクは存在したとしても極めて小さいと常に結論づけてきたこれまでのリスク分析を裏付けている。

報告企業の意見	今後の対応
仏国における vCJD に関するリスク分析情報である。 血漿分画製剤では現在まで感染例は記録されておらず、血漿分画製剤のリスクは存在したとしても極めて小さいと、従来の見解と同様に結論づけている。	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。

Risque de transmission par le sang de la maladie de Creutzfeldt-Jakob : évolution de l'analyse de risque depuis 15 ans - Point d'Information

04/10/2013

Dans un article[1] publié dans le revue Transfusion Clinique et Biologique, issu d'une collaboration entre l'ANSM et la Faculté de Pharmacie de Paris, les auteurs retracent l'évolution en France de l'analyse des risques de transmission de la maladie de Creutzfeldt-Jakob par le sang et ses dérivés, entre 1990 et 2010. Cette revue contribue à comprendre comment les données scientifiques pertinentes ont été exploitées pour construire cette analyse dans une perspective de décision de santé publique.

Le risque de transmission de la maladie de Creutzfeldt-Jakob (agent infectieux transmissible, responsable d'encéphalopathie spongiforme bovine - ESB), par le sang et les produits dérivés du sang (notamment les médicaments dérivés du plasma, tels que les facteurs de la coagulation ou les immunoglobulines) a été au centre des préoccupations des autorités sanitaires dès les années 1980, avec un regain d'intérêt dans les années 1990 avec l'apparition d'une part de l'épidémie de "vaches folles" en Angleterre et les premiers cas de variant de la maladie de Creutzfeldt-Jakob (vMCJ) en 1996.

Les auteurs, Marc Martin (ANSM) et Jean-Hugues Trouvin (Université Paris-Descartes), rappellent comment l'analyse de risque a été conduite par les autorités françaises tout au long de la période 1990-2010, les hypothèses de travail qui ont été utilisées et révisées au fur et à mesure de l'évolution des connaissances scientifiques. A ce jour, 27 cas de vMCJ ont été identifiés en France, et les dernières projections avancent qu'il pourrait y avoir au total une centaine de cas en France sur une période de 60 ans. En ce qui concerne les produits sanguins labiles, le risque de transmission est avéré, mais compensé par le bénéfice attendu d'une transfusion sanguine. En revanche, aucun cas de transmission avec des produits sanguins stables préparés suivant les méthodes de préparation mises en œuvre en France depuis les années 90 n'a jusqu'ici été documenté, ce qui corrobore les analyses de risque conduites depuis les années 2000 qui ont toujours conclu à un risque extrêmement limité avec les médicaments dérivés du plasma, s'il existe.

[1] Risk of transmission of Creutzfeldt-Jakob disease via blood and blood products. The French risk-analysis over the last 15 years

Risque de transmission de la maladie de Creutzfeldt-Jakob par le sang et ses dérivés. L'analyse de risque Francaise au cours des 15 dernières années M. Martin a,*, J.-H. Trouvin a,b Q1
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クロイツフェルト・ヤコブ病の血液による感染リスク：15年間のリスク分析の変遷―“point d'information”（情報欄）より
2013年10月4日

ANSMとパリ大学薬学部の協力から生まれ、Transfusion Clinique et Biologique誌に掲載された論文[1]で、著者らは、1990年から2010年までの血液及び血液製剤によるクロイツフェルト・ヤコブ病の感染リスクの分析の変遷を追跡した。この雑誌は、公衆衛生の意思決定という視点から、このリスク分析を構築するために関連科学データがどのように利用されたかを理解するのに役立つ。

血液及び血液製剤（特に、凝固因子又は免疫グロブリンなどの血漿製剤）によるクロイツフェルト・ヤコブ病（ウシ海綿状脳症（BSE）の原因となる感染性因子）の感染リスクは、1980年代初めから保健当局の関心の中心であり、英国における「狂牛病」の発生、さらに1996年の変異型の最初の患者の発生により1990年代に再度関心が高まった。

著者のMarc Martin（ANSM）及びJean-Hugues Trouvin（パリ・デカルト大学）は、1990年から2010年までの間にフランス当局がどのようにリスク分析を行ってきたかを、科学的知識の発展に伴って使用、修正された作業仮説とともに説明した。現在、フランスでは27例のvCJD症例が同定されており、最新の予測では、フランスにおいて60年間の合計は100例程度になりうるとされている。輸血用血液製剤については、感染リスクは知られているが、このリスクは輸血により得られるベネフィットで相殺される。反対に、1990年代からフランスで採用されている製造法で作られた血漿分画製剤では現在まで感染例は記録されておらず、このことは、2000年代から実施され、血漿分画製剤のリスクは存在したとしても極めて小さいと常に結論づけてきたこれまでのリスク分析を裏付けている。

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
				2013. 9. 6	該当なし	

一般的名称	解凍人赤血球液	研究報告の公表状況		公表国
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社) 解凍赤血球液-LR「日赤」(日本赤十字社) 照射解凍赤血球液-LR「日赤」(日本赤十字社)		Segarra C, Bougard D, Moudjou M, Laude H, Béringue V, Coste J. PLoS One. 2013 Jul 24;8(7):e69632. doi: 10.1371/journal.pone.0069632. Print 2013.	フランス

研究報告の概要

○潜伏期における異常プリオン蛋白(PrP^{TSE})の検出:プラスミノーゲンを用いた分離と増幅技術の併用

背景: 英国で輸血と第VIII因子製剤によるとみられる変異型クロイツフェルト・ヤコブ病(vCJD)感染症例が報告されて以来、血液中にvCJDの感染性が存在する可能性は高いと考えられている。vCJD感染の潜伏期または発症患者の血液検査を効果的に行うには、フェムトモル濃度でPrP^{TSE}を検出する方法が必要である。

方法/主な所見: プラスミノーゲンをコーティングした磁気ナノビーズを用いたPrP^{TSE}の分離、蛋白質ミスフォールディング循環増幅(PMCA)法による増幅及びウエスタンブロット法による検出を行う3段階から成る検査法を開発した。スクレイピー感染脳ホモジネートからのプラスミノーゲン・ナノビーズによるPrP^{TSE}回収率は95%であった。この検査によって、無症候キャリアにおけるPrP^{TSE}の検出に必要とされる感度(フェムトグラム)に相当する、vCJD感染脳ホモジネートの10^{-8}希釈物におけるPrP^{TSE}検出が可能となった。また、NIBSCのコントロールヒト血漿パネルを用いた検査により特異性が100%であることが確認された。

結論/意義: この高感度・高特異性の増幅検査は、vCJD感染の潜伏期にある患者の血漿及びバフィーコートからのPrP^{TSE}の検出を可能にした。この検査は、大規模スクリーニングで陽性反応を示した患者の血液中におけるPrP^{TSE}の存在を確認する検査候補として有望である。

使用上の注意記載状況・その他参考事項等

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

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
プラスミノーゲンを用いた分離と増幅技術の併用による高感度・高特異性のPrP^{TSE}(vCJDを含む)検出法を開発したとの報告である。	日本赤十字社は、vCJDの血液を介する感染防止の目的から、受付時に過去の海外滞在歴を確認し、欧州等38カ国に一定期間滞在したドナーを無期限に献血延期としている。また、英国滞在歴を有するvCJD患者が国内で発生したことから、1980～96年に1カ月以上の英国滞在歴のある人の献血を制限している。今後もプリオン除去フィルター等の技術を含め、CJD等プリオン病に関する新たな知見及び情報の収集に努める。

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Plasminogen-Based Capture Combined with Amplification Technology for the Detection of PrP^{TSE} in the Pre-Clinical Phase of Infection

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Abstract

Background: Variant Creutzfeldt-Jakob disease (vCJD) is a neurodegenerative infectious disorder characterized by a prominent accumulation of pathological isoforms of the prion protein (PrP^{TSE}) in the brain and lymphoid tissues. Since the publication in the United Kingdom of four apparent vCJD cases following transfusion of red blood cells and one apparent case following treatment with factor VIII, the presence of vCJD infectivity in the blood seems highly probable. For effective blood testing of vCJD individuals in the pre-clinical or clinical phase of infection, it is considered necessary that assays detect PrP^{TSE} concentrations in the femtomolar range.

Methodology/Principal Findings: We have developed a three-step assay that firstly captures PrP^{TSE} from infected blood using a plasminogen coated magnetic nanobead method prior to its serial amplification via protein misfolding cyclic amplification (PMCA) and specific PrP^{TSE} detection by western blot. We achieved a PrP^{TSE} capture yield of 95% from scrapie-infected material. We demonstrated the possibility of detecting PrP^{TSE} in white blood cells, in buffy coat and in plasma isolated from the blood of scrapie-infected sheep collected at the pre-clinical stage of the disease. The test also allowed the detection of PrP^{TSE} in human plasma spiked with a 10^{-7} dilution of vCJD-infected brain homogenate corresponding to the level of sensitivity (femtogram) required for the detection of the PrP^{TSE} in asymptomatic carriers. The 100% specificity of the test was revealed using a blinded panel comprising 96 human plasma samples.

Conclusion/Significance: We have developed a sensitive and specific amplification assay allowing the detection of PrP^{TSE} in the plasma and buffy coat fractions of blood collected at the pre-clinical phase of the disease. This assay represents a good candidate as a confirmatory assay for the presence of PrP^{TSE} in blood of patients displaying positivity in large-scale screening tests.

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Introduction

The prion disease variant Creutzfeldt-Jakob (vCJD) was first identified in the UK in 1996 and was shown to represent the human counterpart of bovine spongiform encephalopathy (BSE), a consequence of the entry of contaminated beef products into the human food chain in the 1980s [1]. Up to August 2012, 227 clinical cases had been reported across 12 countries, 176 of which were in the UK alone, 27 in France and 24 in the other 10 countries [2]. Although the incidence of food-borne vCJD is declining, secondary transmission of vCJD through blood transfusion or tissue grafts continues to pose a genuine risk to public health. Several studies have now demonstrated the efficient transmission of Transmissible Spongiform Encephalopathy (TSE) by blood in non-human primates [3] and in sheep [4,5]. Using TSE-infected sheep models, several teams have confirmed that all blood components can transmit the infectious agent through blood transfusion into healthy sheep [5–7]. Moreover, O. Andreoletti et al. demonstrated that the transmission was highly efficient, as 0.2 ml of infected whole blood was sufficient to elicit the disease in sheep [8]. On the issue of transfusion risk, the United Kingdom (UK) reported five secondary cases, four of which (three clinical and one subclinical) were likely associated with the transfusion of non leukoreduced red blood cell concentrates. The fifth case concerned a patient treated with clotting factor FVIII manufactured from the plasma of a donor who developed vCJD six months after donating in 1996. However, the true size of the reservoir of asymptomatic carriers, all of whom represent potential blood donors, remains undetermined. Recently, taking into account the existence of healthy carriers of vCJD, Gharske T. et al predicted the number of vCJD cases associated with secondary transmission in the UK over the coming years will exceed the number of cases

of primary transmission via contaminated food [9,10]. Interim data from a repeat appendix survey have reported 16 positives out of 32441 samples leading to a prevalence of 1 in 2000 persons in the UK population [11]. The introduction of a vCJD detection assay could provide valuable information regarding the true prevalence of pre-clinical cases and help to prevent further cases resulting from blood transfusion transmission.

The major event in this disease is a conformational change of the normal cellular prion protein (PrP^{C}) into an infectious misfolded isoform (PrP^{TSE}) which accumulates as macromolecular assemblies in the brain [12]. At variance with sporadic forms of Creutzfeldt-Jakob disease, vCJD PrP^{TSE} accumulates not only in the brain but also in lymphoid organs [13,14], and is probably present in biological fluids.

A femtomolar level of sensitivity – i.e. 0.1 pg/mL or 10 infectious doses per mL (ID/ml) – has been estimated from the hamster model, as the minimum required to detect PrP^{TSE} in the plasma of a donor at the preclinical phase of infection [15]. This concentration is very difficult to detect using classical PrP^{TSE} detection methods.

Accordingly, we have been focusing our attention on an amplification technology called protein misfolding cyclic amplification (PMCA) developed by C. Soto et al. [16]. In essence, PMCA facilitates the conversion of PrP^{C} substrate in the presence of low or undetectable amounts of PrP^{TSE}, to achieve levels visible by conventional laboratory methods such as immunoblotting. This is achieved by subjecting samples to repeated and alternating cycles of incubation thought to enlarge the PrP^{TSE} seed, and sonication thought to fragment it and generate new catalytic units. A serial PMCA method has been optimized for the high-efficiency amplification of PrP^{TSE} from different animal or human TSE-infected samples [17–22]. However, direct amplification of blood PrP^{TSE} by PMCA has proved difficult, mostly due to the difficulty in obtaining even minute quantities of prions, which generally requires large sample volumes, but also to the presence of blood-associated conversion inhibitors that interfere with the amplification [18,23]. To circumvent this, a pre-analytical step is needed to capture PrP^{TSE} from blood samples, in a specific manner, before amplification. Magnetic beads can be coated with plasminogen which has been reported to bind preferentially PrP^{TSE} from multiple species including sheep and human [24–26]. Plasminogen also has demonstrated the ability to stimulate prion conversion *in vitro* [27].

We designed a specific PMCA-based confirmatory test for the detection of PrP^{TSE} in blood, which comprises three steps: 1) plasminogen-coated nanobeads capture of PrP^{TSE} from blood components; 2) PrP^{TSE} amplification by serial PMCA; and 3) specific detection of PrP^{TSE} by immunoblotting. As the number and volume of vCJD blood samples are very limited in the UK and in France, assay validation was first undertaken on sheep blood samples collected at pre-clinical or clinical stages of scrapie. The sensitivity and specificity of the test were then determined on human vCJD spiked-plasma panels provided by the National Institute of Biological Standards and Control (NIBSC). After optimization of the different steps, this test reached both the 100% specificity and the sensitivity levels required for the detection of infectivity in asymptomatic carriers.

Materials and Methods

Sample Preparations

Brain homogenates were provided by different teams: scrapie-infected transgenic mouse (tg338) brains (127S strain [28], vCJD-infected tg650 mouse brains [29] and null tg mice ($Prnp^{-/-}$) were from INRA (78350 Jouy-en-Josas, France); vCJD infected brain homogenate (IBH) ref 05J18 was from CHU-Lyon (Lyon 69677, Bron-France, France); and vCJD IBH Ref NHBY0/0003 was from NIBSC (Potters Bar, UK). All the animal experiments made to inoculate the mice and collect the brains at euthanasia were carried out in strict accordance with EU directive 2010/63 and were approved by the INRA institution local ethics committee (Comethea; permit number 12/034).

Anonymized human whole blood samples were collected in EDTA collection tubes (Etablissement Français du Sang – Pyrénées Méditerranée). Donor written consent had been obtained for their use in research in compliance with French Law (code de la santé publique article L.1243–3) concerning Blood and Tissue Samples for Non therapeutic Use. Since our study uses plasma only as diluent, it does not belong to the field of IRB competence as defined in the French regulation, and therefore does not require the approval of the Bioethical Review Board. Plasma was then isolated and recovered after centrifugation at 1500 x *g* for 15 min at room temperature (RT).

Whole blood was also collected from healthy (4) and scrapie-infected sheep at preclinical (PG127 isolate at 120 days after oral challenge) (1) or terminal (natural scrapie) (4) stages of the disease. Sheep blood samples were obtained from O.Andreoletti, (Institut National de la Recherche Agronomique/Ecole Nationale Vétérinaire de Toulouse – France) as part of a research agreement. The sheep expressed the V136R154Q171 allele of ovine PrP. Sheep white blood cell (SWBC) samples were prepared from the buffy coat (BC) fraction, obtained after centrifugation at 2000 x *g* for 15 min at Room Temperature (RT). Residual red cells were eliminated from the buffy coat in buffer composed of 155 mM NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA (pH 8). Aliquots of 10^7 phosphate buffer saline (PBS)-cleared SWBC were stored at –80°C.

Spiked plasma samples were prepared as follows: 500 µl of healthy human donor plasma were spiked with serial tenfold dilutions (ranging from 10^{-2} to 10^{-10}) of either vCJD 10% IBH from affected patients or scrapie 10% 127S IBH (127S strain = mouse adapted PG127 isolate) from ovine transgenic mice (*tg338* line).

Prion Protein Capture

The use of human plasminogen as an efficient ligand for the capture of prion proteins has been reported by Fischer *et al.* [24]. Accordingly, we used super para-magnetic nanobeads activated with carboxylic acid functionality (for bead specifications see ref 0211, Ademtech - France), which were coated with human plasminogen (Fluka Sigma-Aldrich – France) by shaking for 2 h at 37°C. The optimal ligand quantity to be used was evaluated by testing three plasminogen concentrations: 10, 20, and 30 µg/mg of beads (10 µg/mg being the lowest concentration recommended by the manufacturer). After a blocking step with 0.5 mg/ml albumin solution at 37°C, beads were stored at 4°C in suspension at 1% (w/v) in the Ademtech storage solution.

Spiked plasma samples (500 µl) were mixed (1:1) with a lysis/ligation buffer (LB), PBS, 3% NP40, 3% Tween 20 before incubation with the plasminogen-coated beads at RT for 90 min.

Firstly, we evaluated the optimal bead quantity to be used for each sample by testing several volumes of 1% bead suspension (2.5, 5, 10, 20, 30, 60 and 90 µl). After washing with PBS, the magnetic beads bearing PrP^{TSE} were isolated and PrP^{TSE} protein bound on beads was amplified by PMCA.

Different volumes of healthy and infected sheep plasma and buffy coat were mixed with the LB buffer (final volume 1 ml)

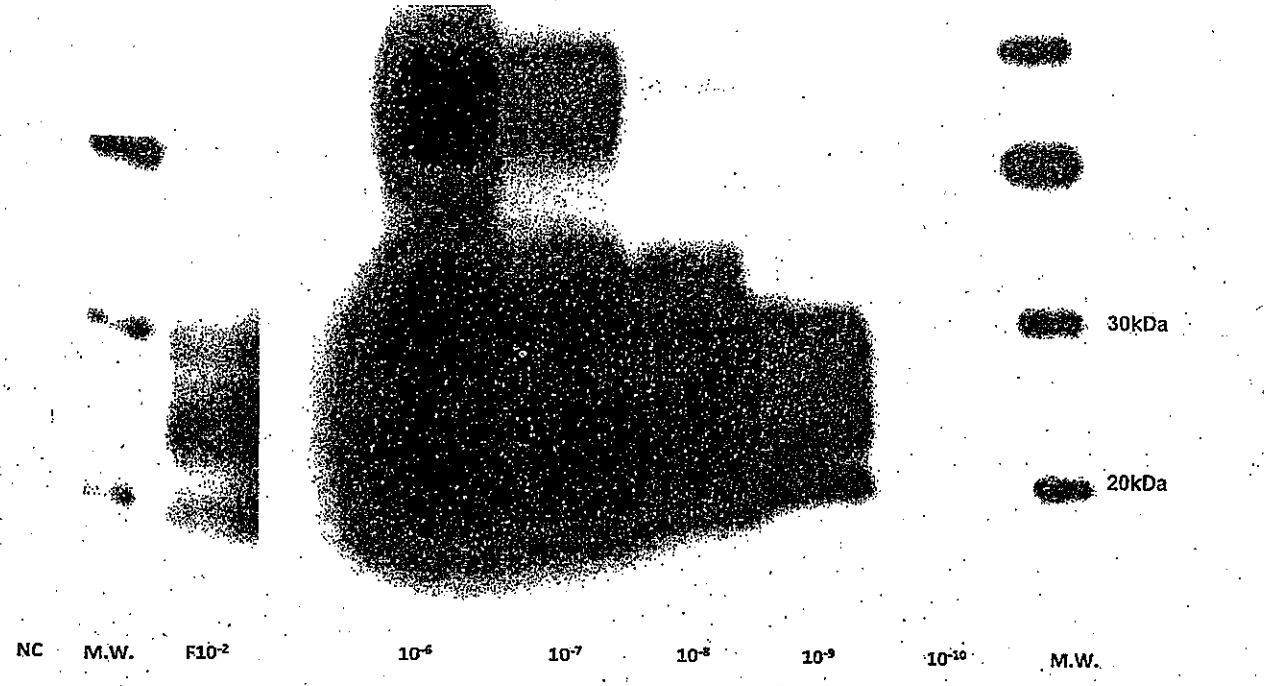

Figure 1. Ovine PMCA optimization. Tg338 127S infected brain homogenate dilutions (10^{-4} to 10^{-10}) were tested using two rounds (one round = 80 cycles) of PMCA and detection was performed on PK-digested amplified products using western blot analysis with 6D11 as the primary antibody. Molecular weight markers are shown on the right. NC: negative control, NBH only. $F10^{-2}$:10^{-2} IBH dilution without PMCA (Frozen).
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before incubation with the determined quantity of plasminogen-coated beads at RT for 90 min as described above.

For PrP^{TSE} capture from SWBC samples, the cells were first incubated on ice for 30 min with LB. After centrifugation at 1000 *g* at 4°C for 2 min the supernatant was harvested and mixed with LB (final volume 1 ml) before incubation with plasminogen-coated nanobeads as described above.

Protein Misfolding Cyclic Amplification (PMCA)

PrP^{C} sources for PMCA were from normal brain homogenates (NBH) from either human PrP (M^{129} allele, tg650 line) [29] or ovine PrP (VRQ allele, tg338 line) [30] transgenic mice. These mice overexpress the level of PrP^{C} by 6 and 8 fold respectively, compared to wild-type mice. Brains were collected and prepared as previously described [21]. Briefly after collection, brains were rinsed in cold PBS and immediately frozen on dry ice before long-term storage at −80°C. Brains were then homogenized, using a potter-Elvehjem homogenizer on ice, at a 10% (wt/vol) concentration in the lysis/converting buffer (CB) composed of 150 mM NaCl, 1%Triton X-100 and protease inhibitor cocktail (Roche) in PBS (pH 7.2). Homogenates were centrifuged at 2000 x *g* for 20 seconds and frozen at −80°C in single-experiment aliquots.

The effect of PrP^{C} overexpression on PMCA efficacy was evaluated by testing varying NBH dilutions: 10% tg338 NBH alone, then 1:6 and 1:8 dilutions of 10% tg338 NBH in 10% PrP−/− NBH [31].

Captured prion protein was first mixed with 90 µl of 10% NBH. Then, amplification by PMCA was performed using the Misonix 4000 (Misonix, N.Y., USA). Each cycle is composed of an incubation step (37°C) and a sonication step. Beforehand, the following PMCA parameters were optimized: incubation duration per cycle (30 and 60 min), sonication duration per cycle (20 and 40 s), power level (60, 70, 80%) and the number of PMCA cycles (50, 80,100) per round.

For sample analysis, after a tenfold dilution of the amplified samples with fresh NBH, a second and a third round of PMCA was performed.

Proteinase K (PK) Digestion and SDS-PAGE/ immunoblotting

Methods were performed as previously described [32]. Briefly, after bead removal, amplified products were incubated at 45°C with PK (200 µg/ml) for 60 min, before their denaturation at 100°C in SDS-PAGE sample buffer. Samples were run on 12% NUPAGE gels and electrotransferred onto PVDF membrane. Western blotting, using the SNAP system (Millipore, St-Quentin-en-Yvelines, France), was performed with 3F4 or 6D11MAb as anti-PrP monoclonal antibodies (MAb) (Signet/Proteogenix, 67412 Illkirch, France) for human and sheep prion detection respectively, and an anti-mouse IgG peroxidase-linked secondary antibody for chemiluminescent reaction (ECL reagent, GE-Healthcare France).

The capture yield was calculated as follows: the amount of PrP^{res} captured by plasminogen-beads was compared to the total amount of PrP^{res} present in the input after acquisition of the chemiluminescent western blotting signals with the GeneGnome digital imager and quantification with the GeneTools software (Syngene, Frederick, Maryland, USA). Three independent experiments were performed.

National Institute for Biological Standards and Control (NIBSC) Panels

The NIBSC has made available a series of reference reagents prepared from autopsied human brain specimens (www.nibsc.ac.

uk/cjd/brainsamples.html). The brain reference reagents used in this study were obtained from the CJD Resource Center in the form of 10% (wt/vol) homogenates in 0.25 M sucrose. Two panels were tested. Panel 1 was composed of ten randomly chosen normal plasma samples distributed in duplicate (a total of 20). Panel 2 was a blinded panel composed of duplicates of vCJD brain dilutions in normal plasma, and negative controls including plasma spiked with normal brain and negative plasma alone (96 tubes).

Results

Optimization of the Plasminogen-based Capture System and Serial PMCA Technology using Scrapie-infected Brain Material

The PMCA parameters were optimized using normal and scrapie-infected brain homogenates from ovine PrP transgenic mice (tg338 line, VRQ allele) in the PMCA reaction. PMCA was first evaluated by comparing cycle numbers, incubation, sonication and power parameters of the Misonix. Finally, after two PMCA rounds of 80 cycles each (30 min incubation, 20 s sonication, 80% power) a PrP^{TSE} specific signal was reproducibly detected up to 10^{-9} dilution (n = 3 from two different IBH) indicating a 7 log amplification compared to the signal obtained for the non-amplified 10^{-2} dilution ($F10^{-2}$) (Fig. 1). No signal was detected when normal brains (about 50 samples throughout this study) were processed similarly from different NBH samples.

We evaluated the effect of PrP^{C} overexpression on the PMCA efficacy of 10^{-5} and 10^{-6} IBH dilutions using three different dilutions of tg338 brain substrates: undiluted 10% tg338 NBH and 1:6 and 1:8 dilutions of 10% tg338 NBH in 10% PrP−/− NBH. Only undiluted 10% tg338 NBH, demonstrated maximum amplification efficacy for both 10^{-5} and 10^{-6} IBH dilutions. Using the dilution representative of 'physiological' expression levels of PrP^{C} found in sheep i.e. 1:8, no signal was obtained for the 10^{-6} IBH dilution and a very faint signal was detected for the 10^{-5} IBH dilution (Fig. 2).

Prior to PMCA, PrP^{TSE} needs to be captured from blood samples and concentrated. The optimum plasminogen concentration and bead volume for PrP^{TSE} capture were firstly calculated using a series of plasminogen-coated nanobeads. Scrapie IBH at 10^{-3} and 10^{-4} dilutions was diluted in human plasma (500 µl) and mixed with various volumes of beads with different plasminogen concentrations before PMCA amplification. Representative results are shown in Fig. 3A and B. It is worth noting that lower signals were observed with increasing bead volume (Fig. 3 B lanes 6–8) and plasminogen concentration (Fig. 3A lanes 3, 4, 5, 7, 8, 9, 10, 11, 12). The strongest PrP^{TSE} signal was observed using a plasminogen (Plg) concentration of 10 µg Plg/mg of beads (Fig. 3A lanes 3) and a volume of 10 µl of the 1% bead suspension per 500 µl of spiked plasma (Fig. 3B lanes 4, 6). Under these conditions, when PrP^{TSE} was captured from 10 µl Tg338 127S IBH or 10 µl vCJD IBH diluted in 500 µl of plasma and analyzed directly by western blot without PMCA amplification, a 95% yield (percentage of recovery calculated from PrP^{res} signal) was observed (Fig. 4). In the same way, the PrP^{TSE} signal obtained after one PMCA round from the 10^{-6} plasma dilution appeared almost similar to that obtained when a control 10^{-6} IBH dilution (Fig. 5 $C10^{-6}$) was used directly to seed the PMCA substrate suggesting that the association of the capture by plasminogen-coated nanobeads and PMCA reached almost 100% efficiency (Fig. 5; lanes 10^{-6} and $C10^{-6}$). Both negative controls of 500 µl of human plasma mixed with plasminogen-coated nanobeads prior to PMCA remained negative (Fig. 5 lanes NC).

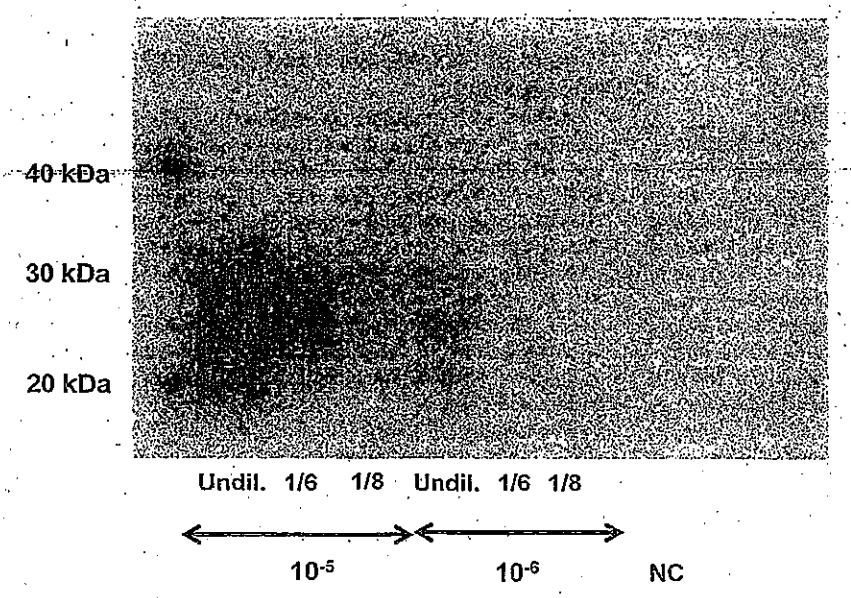

Figure 2. Effect of PrP^{C} level on PMCA efficacy. Tg338 127S infected brain homogenate dilutions (10^{-5} and 10^{-6}) were PMCA amplified (one round) using 10% tg338 NBH (Undil.), 10% tg338NBH diluted 1/6 in 10% tg-$PrP^{0/0}$NBH and 10% tg338NBH diluted 1/8 in 10% tg-$PrP^{0/0}$NBH as PMCA substrates. The detection was performed on PK-digested amplified products using western blot analysis with 6D11 as the primary antibody. Undil.: 10% tg338 NBH without dilution in 10% tg-$PrP^{0/0}$NBH. NC: negative control, NBH only.
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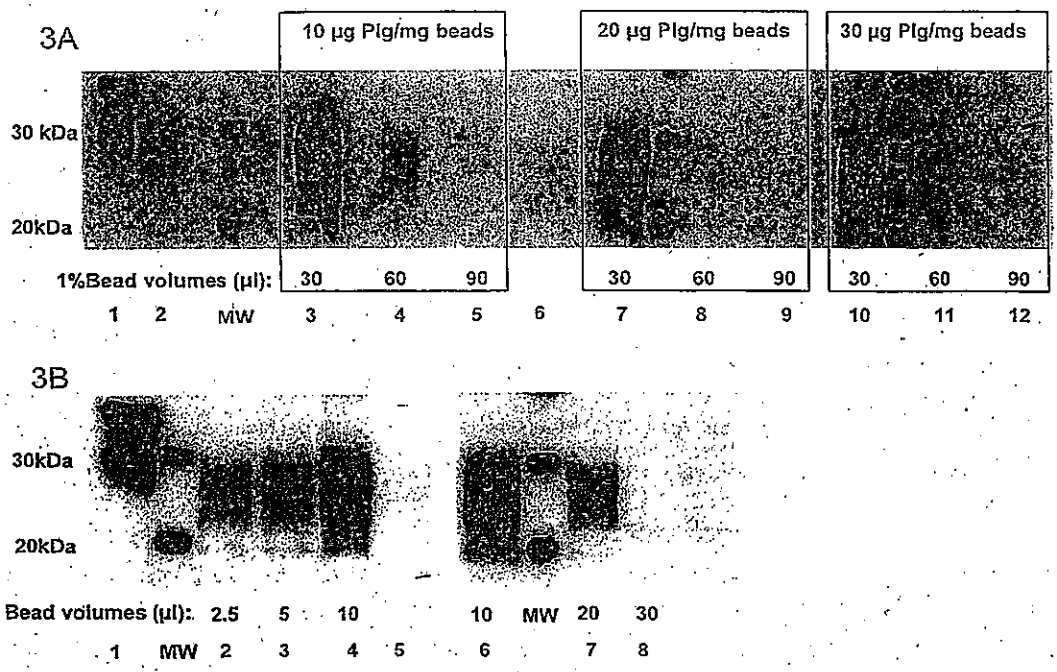

Figure 3. Capture optimization of ovine brain PrP^{TSE}. **3A:** Batches of nanobeads (1% suspension) coated with 10, 20, and 30 µg of plasminogen/mg of beads were used to test prion capture efficacy using 30, 60 and 90 µl of plasminogen-coupled beads per 500 µl of human plasma spiked with a 10^{-3} dilution of 127S IBH. After one round of PMCA, detection was performed on PK-digested and amplified products using western blot analysis with 6D11 as the primary antibody. Lane 1: NBH: normal brain homogenate without PK digestion Lane 2: $F10^{-2}$ IBH dilution without PMCA (Frozen) Lane 6: negative control: plasma only **3B:** Tg338 127S IBH dilution (10^{-4}) in plasma was tested using different volumes of coated beads at 10 µg of plasminogen/mg of beads (1% suspension) for the prion capture. After one round of PMCA, detection was performed on PK-digested and amplified product using western blot analysis with 6D11 as the primary antibody. Lane 1: NBH: normal brain homogenate without PK digestion Lane 5: negative control: plasma only.
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Validation of the Plasminogen-coated Nanobeads - sPMCA Combined Assay on a Panel of Blood Samples from Healthy and Infected Sheep

We next examined whether our experimental conditions could specifically detect PrP^{TSE} in scrapie-infected blood samples.

In four white blood cell concentrates of naturally scrapie-infected sheep (SWBC), 0/4 and 1/4 samples were detected positive respectively after one and two rounds of PMCA (results not shown). After a third round, a PrP^{TSE} signal was obtained from all four infected SWBC amplified samples, in contrast to those from healthy sheep (0/4) (Fig. 6). These results were

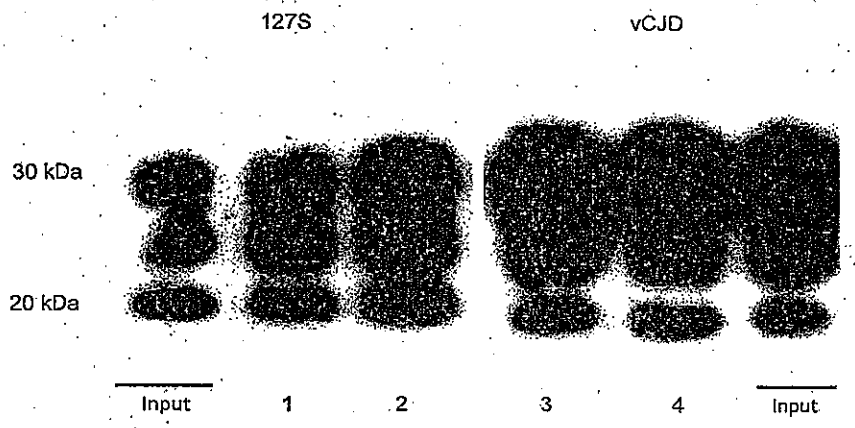

Figure 4. Efficacy of PrP^{TSE} capture. Ten microliters of 10% 127S (lanes 1 and 2) and vCJD IBH (lanes 3 and 4) were diluted in 500 µl of plasma and incubated 2 h with plasminogen-coated beads. PrP^{TSE} bound to the beads were PK-digested and denatured in sample buffer for western blot analysis with 6D11 and 3F4 anti-PrP MAbs. Percentage yield was quantified with Genetools software after acquisition of the chemioluminescent western blot signals with the Genegnome digital imager (Syngene, US) Lanes 1 and 2:127S IBH capture Lanes 3 and 4: vCJD IBH capture.
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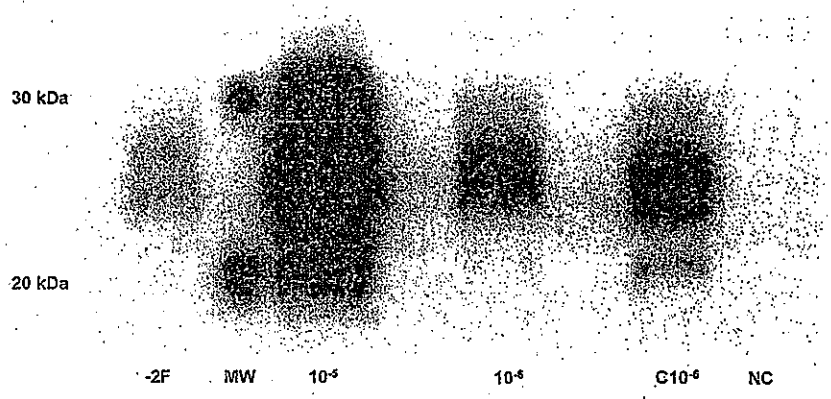

Figure 5. Sensitivity of the ovine PrP^{TSE} optimized test. Tg338 127S IBH dilutions (10^{-5}, 10^{-6}) in 500 µl of human plasma were captured with 10 µl of coated nanobeads at 10 µg of plasminogen/mg of beads, amplified by PMCA (one round of 80 cycles) and detection was performed on PK-digested and amplified products using western blot analysis with 6D11 as the primary antibody. −2F: 10^{-2} IBH dilution without PMCA (Frozen) C10^{-6}: IBH dilution amplified directly by PMCA without capture step NC: negative control analyzed along with samples.
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reproduced in three different assays. In one sheep at the preclinical phase of scrapie, a specific PrP^{TSE} signal was detected in a 500 µl plasma sample after PrP capture and two rounds of PMCA (Fig. 7A) and in both 50 µl and 25 µl buffy coat samples (BC) after one PMCA round (Fig. 7B). These assays were reproduced twice and each sample was tested in duplicate.

Evaluation and Validation of the Plasminogen-coated nanobeads/sPMCA Assay on Human Samples

Similar PMCA optimizations were performed on vCJD human brain homogenates amplified with normal brain homogenates from human PrP transgenic mice (M^{129} allele, tg650 line) as substrate. The optimal amplification parameters were found to be exactly the same for the human vCJD strain as for the ovine strain (80 cycles: 30 min incubation, 20 s sonication, 80% power).

After the PrP^{TSE} capture from a panel of tenfold dilutions of vCJD IBH (10^{-4} to 10^{-8}) in normal human plasma, the first PMCA round allowed the detection of PrP^{TSE} at the 10^{-5} dilution. This indicated a 3 log amplification compared to the signal obtained for the non-amplified $F10^{-2}$ dilution (Fig. 8). After a tenfold dilution of the amplified samples with fresh NBH, a second and a third round of 80 PMCA cycles was performed allowing detection of PrP^{TSE} up to 10^{-6} and 10^{-8} dilutions respectively, corresponding to a 4 and 6 log amplification factor (Fig. 8). These sensitivity levels were confirmed three times with different IBH samples, one of which was the WHO reference reagent provided by NIBSC. It is worth noting that this 10^{-8} dilution detection after the third round was the same as that obtained when testing tenfold dilutions of vCJD IBH (10^{-4}–10^{-8}) without the capture step (results not shown); this confirms a capture efficacy around 95% as shown in fig. 4.

The analytical performance of the test was then evaluated in terms of its sensitivity and specificity by testing blinded panels provided by the NIBSC. All samples from panel 1 (normal plasma) tested negative (20/20), demonstrating the test's 100% specificity. Results of the 96 spiked-plasma samples of blinded panel 2 (spiked with either brain or spleen homogenates) obtained after three rounds of PMCA were analyzed by the NIBSC and a sensitivity level of 10^{-5} vCJD brain homogenate in plasma was achieved (Table 1). For plasma spiked with spleen homogenate the 10^{-1} dilution was detected (4/4). The 36 control samples included in the same panel all tested negative. Results obtained on the 56 negative controls included in panel 1 and panel 2 confirmed the 100% specificity of the assay (56/56).

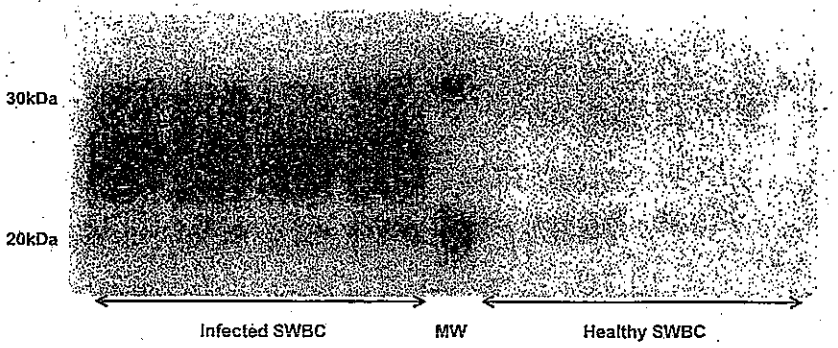

Figure 6. Detection of PrP^{TSE} from SWBC of scrapie-infected sheep. Four infected and healthy SWBC (sheep white blood cells) samples underwent the capture step with 10 µl of coated beads at 10 µg of plasminogen/mg of beads. After three rounds of PMCA, PrP^{res} detection was performed after PK digestion of the amplified products, using western blot analysis with 6D11 as the primary antibody.
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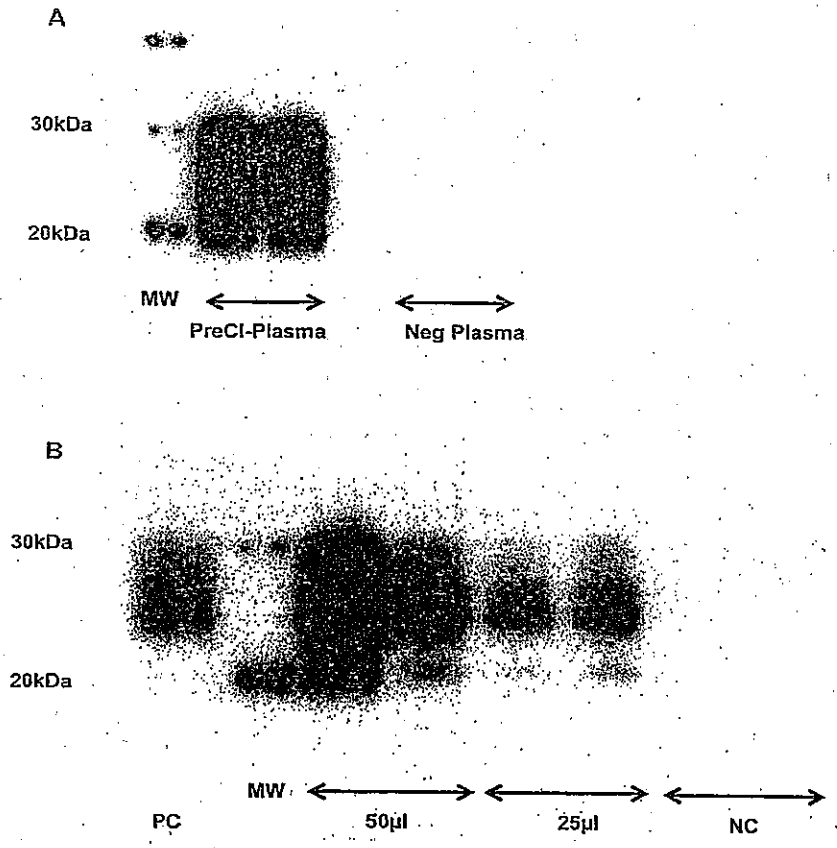

Figure 7. Detection of PrP^{TSE} from plasma and buffy coat of scrapie-infected sheep at the preclinical stage 7A: 500 µl of PG 127 infected and healthy plasma samples underwent the capture step in duplicate with 10 µl of coated beads at 10 µg of plasminogen/mg of beads, before PMCA amplification using two rounds of 80 cycles. Detection was performed after PK digestion of the amplified products using western blot analysis with 6D11 as the primary antibody. Pre-Cl-Plasma: plasma sample from PG127 infected sheep at the pre-clinical stage (120 days post oral challenge) Neg Plasma: plasma sample from healthy sheep **7B:** 50 and 25 µl of PG 127 infected (120 days post oral challenge) and healthy BC (buffy coat) samples underwent the capture step in duplicate with 10 µl of coated beads at 10 µg of plasminogen/mg of beads, before PMCA amplification using one round (i.e. 80 cycles). Detection was performed after PK digestion of the amplified products, using western blot analysis with 6D11 as the primary antibody. Lane PC: 10^{-7} IBH dilution amplified by PMCA Lane NC: Buffy coat from healthy sheep.
doi:10.1371/journal.pone.0069632.g007

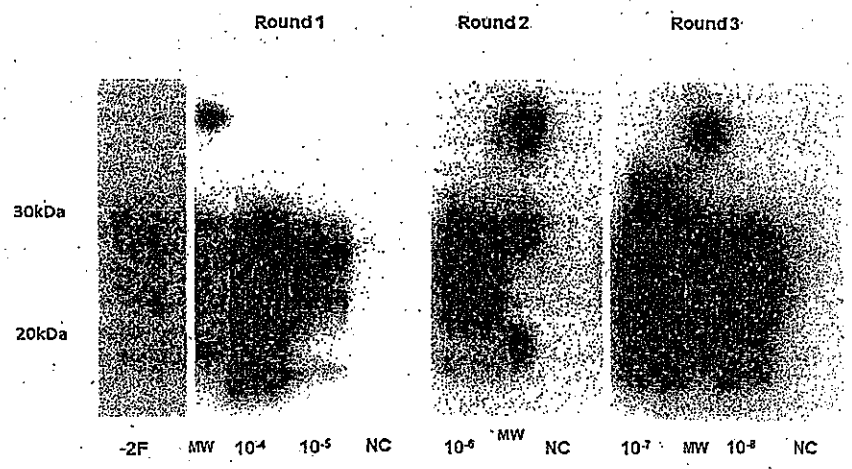

Figure 8. Human PMCA optimization. vCJD IBH dilutions (10^{-4} to 10^{-8}) in 500 µl of plasma were captured by 10 µl of coated beads at 10 µg of plasminogen/mg of beads. After three PMCA rounds (80 cycles/round) the detection was performed on PK-digested and amplified products using western blot analysis with 3F4 as the primary antibody. −2F: 10^{-2} IBH dilution without PMCA (Frozen) NC: negative control: plasma only.
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Table 1. Evaluation of the PrP^{TSE} detection assay on human blinded panel (NIBSC).

Biological materials	Dilutions	Positive
vCJD brain spiked in plasma	10^{-2}	4/4
	10^{-3}	4/4
	10^{-4}	4/4
	10^{-5}	3/8
	10^{-6}	1/4
	10^{-7}	0/4
	10^{-8}	0/8
vCJD spleen spiked in plasma	10^{-1}	4/4
	10^{-2}	0/4
	10^{-3}	0/8
	10^{-4}	0/4
	10^{-5}	0/4
Control brain spiked in plasma	10^{-2}	0/4
	10^{-3}	0/4
	10^{-4}	0/4
Control spleen spiked in plasma	10^{-1}	0/4
	10^{-2}	0/4
Control Plasma	-	0/16

All the controls were collected from individuals showing no sign of vCJD (brain, spleen and plasma).

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Discussion and Conclusion

Results of this study demonstrate that the above-presented three-step PMCA assay can specifically detect PrP^{TSE} at the preclinical stage of the disease in both plasma and buffy-coat samples from sheep. PrP^{TSE} detection achieved after PMCA has previously been described using buffy coat fractions from rodents [33,34] and purified white blood cell preparations from sheep [22] at the terminal stage of the disease. However, PrP^{TSE} amplification efficiency was compromised when the PMCA was performed directly on blood [18]. In a recent study, Tattum et al. [23] showed that when PMCA was performed directly on the whole blood of infected mice, the yield was reduced by approximately 50% because of the presence of inhibitory factors in the blood. To overcome this issue and decrease the consequential large input volumes of blood in the PMCA reaction, we developed a capture technology using plasminogen- a well-known prion protein ligand [24]- for implementation prior to PMCA amplification.

The specificity of PrP^{TSE} capture by plasminogen is controversial. Using high resolution ultrasonography to study interactions between proteins, Negredo et al. [26] demonstrated that the interaction between plasminogen-coated beads and PrP^{TSE} was specific. In addition these findings confirmed previous results obtained by Fisher *et al.* and Maissen *et al.* [24,25]. Conversely however, other studies have reported that recombinant prion proteins and PrP^{C} purified from sheep or bovine brain could also interact with human plasminogen [35–37]. When coupling the capture step with PMCA, our results do not allow us to rule out the possible capture of PrP^{C} isoforms from infected sheep WBCs in addition to PrP^{TSE} by the plasminogen coated beads. However, residual PrP^{C} in addition to PrP^{TSE} does not seem to be a major problem for PMCA, since PrP^{C} is the main substrate for PrP^{TSE} amplification. One limitation could be the potential competition between PrP^{C} and PrP^{TSE} for capture on the covered nano beads. However, in this work, we have demonstrated no loss of PrP^{TSE} during the capture step which allowed 95% capture of the sample PrP^{TSE} before amplification.

We chose nanobeads over microbeads as the support for plasminogen [24] for their small size (100–140 nm) and correspondingly large surface-to-volume ratio [38]. We have previously demonstrated the need to strictly define the nanobead volume to be used for the capture in order to avoid a crowding effect due to an excess of coated nanobeads. Such molecular crowding affects the chemical reactions occurring in high solute concentrations since the volume occupied by one molecule is made unavailable to other molecules and so-called the excluded volume. Theoretical and experimental studies have demonstrated that the excluded volume effect is dependent on the size of molecules (39–41). Thus, the decrease in signal observed when the capture step is performed with an excess of coated beads could be explained by the high molecular weight of the plasminogen (88kDa) fixed on the beads.

In two independent trials, the addition of beads or plasminogen to the PMCA reaction stimulated the PMCA amplification [27,42]. By optimally combining the two enhancement strategies, we have achieved the sensitivity required for PrP^{TSE} detection at the preclinical stage.

The plasminogen-based capture/sPMCA assay applied to scrapie-infected brains from ovine PrP transgenic mice (127S) allowed the detection at a 10^{-9} dilution, equivalent to 100 fg of 127S infected tg338 brain. This represents a 100-fold higher sensitivity level compared to that obtained by bioassay [43]. This finding is not unprecedented as PMCA detection levels well below infectivity levels have been reported for 263K prions in hamsters and chronic wasting disease (CWD) prions in transgenic mice for cervid PrP [20,44].

Applied to WBC samples from naturally infected sheep (n = 4), this assay showed 100% specificity and sensitivity. Moreover, we observed that a capture step is essential to detect PrP^{TSE} in infectious blood samples. Indeed, when the same infected SWBC samples were tested by PMCA alone, the four samples gave negative results (data not shown). Recently, Lacroux et al. reported PrP^{TSE} detection in SWBC collected from sheep experimentally infected by the PG127 scrapie isolate from 90 to 190 day post inoculation (dpi) [7]. In this study, we confirmed the detection of PrP^{TSE} in sheep WBC samples from naturally infected sheep by using the same PMCA substrate (ovine transgenic mouse g338) as Lacroux et al. Therefore, we demonstrated that our plasminogen-based capture allows the detection of two phenotypically different sheep strains. It is worth noting that until now, PMCA detection of the pathological PrP in infected blood samples of animals was obtained using substrate and blood samples of close origins (species, strains and genotype) [21,22,45].

Finally this new assay can detect PrP^{TSE} in a volume of buffy coat (BC) as low as 25 µL (around 0.5 ml of whole blood) and in 500 µl of plasma samples, collected from one sheep at the preclinical stage of scrapie (PG 127 isolate). This assay now needs to be repeated with additional sheep samples.

Coupling the capture step to PMCA also allowed the detection of PrP^{TSE} in the 10^{-8} dilution of vCJD IBH spiked plasma (equivalent to 1 pg of initial brain sample). The assay reached a 100-fold higher level of sensitivity compared to that obtained in the PMCA study using platelets as substrate [46]. Based on our findings that the concentration of PrP^{C} has an influence on the PMCA amplification ratio, this improvement in sensitivity may be

explained by the use of tg650 NBH which overexpresses PrP by 6-fold when compared to platelet homogenate.

When the NIBSC panel was tested by our PrP^{TSE} capture followed by three rounds of PMCA, the detection limits achieved were 10^{-5} dilution of vCJD brain spiked in plasma (3/8) and 10^{-1} dilution of vCJD spleen spiked in plasma (4/4). It is worth noting that the 10^{-6} dilution of vCJD brain was also detected in the same experiment but only in one sample out of the four tested. Since the sensitivity of our assay (fig. 8) was checked with vCJD brain homogenates from three different sources, this discrepancy might be explained either by differences in the procedures of material preparation or the infectivity levels of the brains at the time of collection. Furthermore, among six diagnostic assays involving the same blinded NIBSC panel, none have overcome this 10^{-5} detection limit with a 100% specificity [47].

Recently two blood test prototypes were published which allow the detection of endogenous PrP^{TSE} in blood. Both are based on the combination of promising technologies for high throughput testing: i) a solid-state binding matrix to capture and concentrate the PrP^{TSE} coupled with a direct immuno-detection allowing the detection of 71% samples of confirmed clinical vCJD patients [48]; and ii) an immunoprecipitation approach coupled to the quaking-induced conversion (eQuIC) technology which allows the detection of PrP^{TSE} in the blood of hamsters at the preclinical stage of the disease [49].

The macromolecular structure and resistance to proteolysis of circulating PrP^{TSE} as well as the distribution of infectivity among human vCJD blood compartments remain unknown. Based on rodent models, it has been estimated that blood PrP^{TSE} concentrations at the preclinical phase of the disease could be around the femtomolar level, i.e. 0.1 pg/mL [50]. Recently, the PrP^{TSE} concentration in scrapie–affected hamster brain at the clinical phase was estimated by quantitative PMCA to be around 10^{-5} gram/gram of brain [51]. Accordingly, the test developed here with 1pg IBH sensitivity could feasibly allow the detection of 10^{-17} g of PrP^{TSE} (~180 molecules) and achieve the limit of detection (LOD) required for the detection of PrP^{TSE} in vCJD plasma samples at the pre-symptomatic phase.

The next step will be validating our new assay on buffy-coat and plasma samples from individuals with confirmed clinical vCJD, however such samples are very limited in volume and in quantity. Fortunately, the test can overcome the problem of sample volume since it has been shown to be suitable for PrP^{TSE} detection in a buffy-coat volume as low as 25 µl.

The last important issue will also be to determine whether or not amplified vCJD PrP^{TSE} is infectious and if the amplification of PrP^{TSE} correlates with the amplification of infectivity. These studies are ongoing.

In conclusion, we have developed a new highly specific and sensitive in vitro assay which may represent a good candidate for use as a confirmatory PrP^{TSE} detection assay in plasma or buffy coat samples. This assay could ensure that blood donations detected positive by means of a large scale high-throughput screening test are indeed true positive.

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Author Contributions

Conceived and designed the experiments: CS JC. Performed the experiments: CS MM DB. Analyzed the data: CS VB JC. Contributed reagents/materials/analysis tools: CS MM. Wrote the paper: CS DB MM VB JC.

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別紙様式第２－１

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
一般的名称	－		研究報告の公表状況	BMJ 2013;347:f5994 doi:10.1136/bmj.f5994	公表国 英国	
販売名（企業名）	－					

研究報告の概要

変異型クロイツフェルト・ヤコブ病（vCJD）は牛海綿状脳症又は狂牛病のヒト型であり、プリオン蛋白の異常型によるものであると広く認められている。
牛海綿状脳症はピーク時の英国（1992 年）では 36000 頭/年以上の発症があった一般的な疾患であるが、vCJD は現在まで英国における報告は 177 例、他国で 51 例（うち仏国で 27 例）であり、また、過去 2 年間の発症は 1 例のみと稀な疾患である。
vCJD においても末梢組織や血液からの感染拡大が懸念され、感染後期のドナーから製造された血液成分や血液由来製剤による感染が確認されている（手術による感染報告はない）。
英国では、血液供給の安全性を高めるため（血液の白血球除去、特定患者の除外、血液由来製剤の原料血の国外からの供給）や、手術機器からの水平感染のリスクを低減するための対策を行っている。これらの措置の必要性や費用対効果は、英国内に vCJD プリオンの既感染者がどの程度いるのかによる。血液検査法がないため、手術で切除された虫垂および扁桃腺を対象とした人口有病率調査が実施され、扁桃腺の感染はなく、虫垂の感染は 1 人/4000 人で、他の精査された調査では 1 人/2000 人であった。年齢や地域による差、感受性のあるジェノタイプは確認されなかった。
発症患者の疫学調査との不一致があることから虫垂に沈着したプリオン蛋白が疾患特異的でない可能性を考慮し、牛海綿状脳症の発生前の 1970 年以前の虫垂検体の調査が進行中である。もし、vCJD 感染が広範であれば、予防的措置は長期継続されるであろう。被感染者が次の感染源になり得ることから、CJD 患者からの受血者や手術機器が使用された人の献血を禁止し、手術には特別な対処が必要とされる。
多くの重要な疑問が残されているが、英国における専門性のあるプリオン研究のためのキャパシティは十分に配置されている。

報告企業の意見	今後の対応
英国で行われた虫垂の感染調査は2,000～4,000人に１人で疫学調査の結果と不一致であることから牛海綿状脳症の発生前の1970年以前の虫垂検体調査が進行中とのことで、vCJD感染が広範であれば、予防的措置は長期継続されるとの情報である。 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。	今後ともvCJDに関する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。

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EDITORIALS

How widespread is variant Creutzfeldt-Jakob disease?

The disease seems rare but "infection" may be relatively common

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Variant Creutzfeldt-Jakob Disease (CJD) is the human form of bovine spongiform encephalopathy or "mad cow disease." It is one of the family of mainly neurodegenerative diseases known as spongiform encephalopathies because of their histological appearance. These diseases afflict animals and humans and are widely accepted as resulting from the toxic build-up of an aberrant form of a normal cellular protein, the prion protein. Bovine spongiform encephalopathy was common, with more than 36 000 cases in the peak year of the cattle epidemic in the United Kingdom (1992).[1] However, variant CJD has remained mercifully rare, with 177 cases in the UK to date (51 in the rest of the world, 27 of which were in France), and only one in the past two years.[2]

So, is variant CJD yesterday's news? The linked paper by Gill and colleagues (doi:10.1136/bmj.f5675) helps make clear why this is not the case.[3] Sporadic CJD, the "usual" form of CJD, was first described early last century and is found worldwide, with an annual incidence of around 1/1 000 000 population. Prion infectivity is notoriously difficult to inactivate and sporadic CJD had been shown to be transmissible by neurosurgery in case studies published as long ago as 1974. Transmission can also occur by injection or implantation of infected material derived from the central nervous system, as in the epidemic of CJD in recipients of human growth hormone derived from cadaveric pituitaries.[4]

In variant CJD, there are also concerns about spread from peripheral tissue and blood because disease related prion proteins have been demonstrated in lymphoreticular tissue.[5] Variant CJD has been transmitted by blood components and products from donors who later developed the disease, although a convincing case of transmission of variant CJD by surgery has not been documented.[6]

UK health agencies have taken several costly steps to secure the blood supply (leucodepletion of blood, exclusion of certain donors, and sourcing of blood products from outside the UK) and to reduce any risk of horizontal transmission by surgical instruments.[7] How necessary, or cost effective, these measures are depends mainly on how many people in the UK are "infected" with the variant CJD prion. Blood tests in specialist settings have been described,[8] but a test (ideally two tests) that could be used widely for diagnosis and screening remains elusive and would transform the approach to the problem.

In the absence of a blood test, anonymised population prevalence surveys using archived tissue from appendicectomies and tonsillectomies were carried out. Although abnormal prion protein was almost entirely absent from tonsils,[9] a previous survey of appendixes suggested a prevalence of 1/4000.[10] Gill and colleagues in their painstaking examination of more than 30 000 appendix samples arrive at a prevalence of 1/2000, the same order of magnitude. Unlike in clinical cases of variant CJD, no particular age group or geographical region was affected, and no susceptible genotype was identified. In the UK, patients with variant CJD have a modal age at death of 28 years and are diagnosed more often in the north of England and in Scotland. Confirmed cases have all been methionine homozygous (MM) at codon 129 of the gene encoding the prion protein (*PRNP*).[11] It is possible that abnormal deposition of prion protein in the appendix is simply a non-specific finding, so appendicectomy tissue from the 1970s and earlier, before bovine spongiform encephalopathy appeared, is being examined.

If "infection" with variant CJD prion proteins is common then precautionary measures are likely to be in place for a long time, and clinicians need to understand the logic behind them. Clinicians may encounter people deemed, in the words of UK public health agencies, to be "at increased risk" of CJD.[7] These are people who have received blood from someone with CJD or been operated on with surgical instruments that have been used on someone with CJD. The chance of these people having acquired the disease is thought to be great enough that they could, in turn, transmit the disease themselves. They are thus banned from donating blood and special arrangements need to be made for surgery that involves tissues in which prion proteins might be found. Advice from local public health or infection control teams should be sought. Local teams will also probably wish to seek more expert help, usually through the CJD Section of the National Centre for Infectious Disease Surveillance and Control of Public Health England that acts as a clearing house for queries and can link them with the UK's various specialist clinical and research teams.

Although we know much about these fascinating, if terrible, diseases, particularly at the protein chemistry and cellular level,

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many important questions remain. What is the disease phenotype and natural course of variant CJD in genotypes other than MM? What other animal prion diseases may be zoonotic? The replication mechanisms first seen in prion proteins have now been identified in other proteins involved in other common neurodegenerative diseases, including Aβ, amyloid-β in Alzheimer's disease, α-synuclein in Parkinson's disease, and tau in several different conditions.[12] How often, if ever, are any of these transmissible? The UK's prion research capacity with expertise in human and veterinary disease surveillance and pathology, as well as animal facilities for transmission experiments, is well placed to answer such questions. Further disinvestment would be premature.

Competing interests: I have read and understood the BMJ Group policy on declaration of interests and declare the following interests: From 2007 until its dissolution in 2011, I was a member of the UK's Spongiform Encephalopathy Advisory Committee (SEAC) and I have been a member since 2011 of the Advisory Committee on Dangerous Pathogens. Both these independent scientific advisory groups took an active interest in this work and encouraged the UK government to fund work on the prevalence of spongiform encephalopathies.

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研究報告の概要

緒　　言

英国とその他のいくつかの国々での変異型クロイツフェルト・ヤコブ病（vCJD）は、その国の人々がウシ海綿状脳症（BSE）プリオンに暴露されたことが原因である。英国では多数の人々が1980年代後半から1990年代前半に食物連鎖中の汚染された肉製品を介してBSEプリオンに暴露された。vCJDは他の形態のクロイツフェルト・ヤコブ病とは異なり、異常プリオンタンパク質（PrP）が、死亡前では扁桃生検組織検体中に、また死亡後ではリンパ網内系全般とその他のいくつかの組織中に検出されている。リンパ網内系の濾胞性樹状細胞中への異常PrPの蓄積が中枢神経系への侵入に先立って起こるものと考えられており、これまでに行われた観察研究では、臨床症状発現前に手術で摘出され保存されていた検体の中に異常PrPの存在が確認されている。現在までのところ英国で発生したvCJD症例は177例にすぎないとはいえ、vCJD感染の無症候キャリア一状態が存在しているものと思われ、特に低プリオン量での暴露が広範に起こった後に、動物種のバリアを越えて起こっている可能性がある。英国では、異常PrPの出現の調査が現在までのところ4件報告されている(補遺の表1を参照)。これらの調査の結果でも不確実な状況が続いていたため、2008年にSEAC(Spongiform Encephalopathy Advisory Committee, 海綿状脳症諮問委員会、この組織は2011年にTSE Risk Assessment Subgroup of the Advisory Committee on Dangerous Pathogensが取って代わった)は、保存されていた虫垂検体の第2回目の免疫組織化学的調査を行うことを勧告し、臨床症状のない異常プリオン感染の発生についてのいくつかの推定値に差があることの理解を深め、より広範囲の人々が感染しているかどうかを調べることとした。本論文はその2回目の調査で明らかとなった知見に関する報告である。

方　　法

検体収集、匿名化、およびデータハンドリング

1941-1960年生まれのコホートおよび1961-1985年生まれのコホートから、少なくとも30,000件の状態の良い虫垂検体をスクリーニングするために、Health Protection Agency（2013年4月1日以降はPublic Health Englandの一部となった）は2000年から2012年までの間に手術時に摘出された40,000件の虫垂検体を集めた。これらのソースとなる虫垂組織は41カ所の参加病院（補遺の表4参照）で標準的な方法で組織標本のワックスブロック（ホルマリン固定、パラフィン包埋）とされて保管され、Public Health Englandに送付された。今回協同した病院の多くは、前回の虫垂と扁桃の調査にも参加して2000年度より以前の虫垂摘出術で得られた検体を提供しており、今回も追加検体を提供することに同意した。検体が全国をよりよく代表する様な分布となるようにするため、前回の調査で参加病院の少なかった地域では、病院を追加すべくコンタクトし、病院の病理組織部門の長に協力を求めた。

Public Health Englandでは、1症例あたり1個のブロックを選択し、そのブロックを、50件の虫垂検体を集めたもの("bin":容器)を、University College London Institute of NeurologyとAnimal Health and Veterinary Laboratories Agencyの2カ所の協力プリオンスクリーニングラボに渡し、そこで切片化、染色、および専門家による顕微鏡観察が行われた。切片作成と染色についての品質アセスメントの後、検体の各容器はPublic Health Englandのラボに返却された。検体と個人の関連付けがなされず匿名性がしっかりと保たれる不可逆的な方法を完了した後に、検査開始についてスクリーニングラボに許可が出された（図1）。Public Health Englandのコーディネートラボで、我々は調査データベースを作成し、そのデータには、調査番号、症例の性別、5年間の出生年度コホートのどれに属するか、虫垂摘出術が行われた病院の位置する地理的エリアの情報を含めた（図1、表1、および補遺の表4を参照）。発生推定値について2項信頼区間を正確に計算し、サブグループ間の

使用上の注意記載状況・その他参考事項等

代表としてテタノブリンIH静注250単位の記載を示す。

2. 重要な基本的注意

(1)略

1)略

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

発生率の比較はFisherの正確確率検定を用い、有意水準を5%として比較した。

切片の調製と異常PrPの免疫組織化学的検出
各パラフィンブロックから、3枚の切片を第1セットとして切り出した。1枚の切片についてはマウスモノクローナル抗PrP抗体KG9を用い、もう1枚の切片にはICSM35(10,12,13)を用いて（補遺の方法の項を参照）、ペルオキシダーゼ-ジアミノベンチジン検出キット（DAB Map Ventana Medical System; Roche, Burgess Hill, UK）((図2 A-D, F1)で異常PrPを検出した。疑いのある症例を調べて陽性例を確定するために、さらに別の切片と別の抗PrPモノクローナル抗体（3F4と12F10、補遺の方法の項参照）について染色を再度行った（図2, P-S）。2カ所のラボは双方とも同一の装置（Ventana Discovery XT ; Roche Burgess Hill, UK）、試薬、およびプロトコールを用いた。また、半数の検体についてはヘマトキシリン-エオジン染色をも行い、炎症によって影響を受けた比率を調べた。各切片のセットを、5つ以上の濾胞がないか確認し、その組織に虫垂生検組織もしくは誤って採取されたその他の組織が含まれていないか調べた。虫垂検体の他に、リンパ濾胞を含んだ虫垂以外の組織も時に見出されたが、そのような組織については、判断基準に合致していた場合には研究対象とした。濾胞性の免疫反応の可能性のあった検体全てから、さらに12個の4μm切片と3個の10μmパラフィンロールを作成した（図1）。赤色の顆粒球性（化膿性）炎症が存在すると二次濾胞中の濾胞樹状細胞の検出にどのように影響するか調べるために、判定基準（≧5つの濾胞が存在する）に合致した虫垂、特に濾胞が部分的に顆粒球によって圧倒(overrun)されている場合には、CD21免疫染色（これは濾胞樹状細胞のマーカーとして確立されている）を、炎症の程度が非常に軽度、中等度、もしくは重度のもの各々5検体について行った（補遺の図1を参照）。

専門家による顕微鏡観察
不可逆的に匿名化した後、UCL Institute of Neurology (SB) またはAnimal Health and Veterinary Laboratories Agency (MMS/YS) で専門家が検査して検体を「陽性」、「疑わしい」、「非特異的」、もしくは「陰性」にカテゴライズした。「陽性」検体とは、濾胞の肺中心内に濾胞性樹状細胞ネットワークに特徴的な免疫標識を示し、少なくとも1つの濾胞が免疫陽性の濾胞性樹状細胞の小さなネットワークを有していなければならない、とした。陽性を示す濾胞性樹状細胞が同じ濾胞中の連続した切片中に存在するか、またはより深部の切片上の異なる濾胞に存在していなければならない。「疑わしい」とカテゴライズされる検体とは、PrP陽性の濾胞性樹状細胞での典型的なパターンとは一致しないが濾胞中に弱い免疫反応性が認められるか、または連続する次の切片上での再現性はないが弱くはっきりしない反応性の染色が見られるかのいずれかの場合である（図 K, M）。「非特異的」と分類される検体とは、濾胞中心ーたとえば劣化した組織中に見られるものなどに抗体結合が認められるもので典型的な濾胞性樹状細胞の形態を示さないものである（図3 J, L, N）。「陰性」検体とは、免疫反応性を示さなかったものである；濾胞内の非濾胞性の樹状細胞、例えばマクロファージが特異的に標識され、濾胞内部は非特異的に（バックグラウンドとして）標識されるか、または濾胞外部の構造物が標識される（補遺の図1を参照）。次いで我々は調査対象となる切片（「陽性」および「疑わしい」免疫標識の認められた切片）のセットの全てを最初に調べた専門家とは異なる専門家（JWIおよびDH）に渡し、2回の独立の検査を行ったが、それらの検査はNational CJD Research and Surveillance Unitにあるスペア切片の染色を含むものであった（図3C）。最後に3回のミーティングで病理組織の専門家たちが、各報告書とスライドセット（切片セット）をレビューし、組織所見についてのコンセンサスが得られるようにした。

PRNPコドン129遺伝子型の決定
我々は、Minor Groove Bindingプローブを用いたアレル識別によって、陽性検体およびその他のものから選んで、それらのコドン129の遺伝子型を決定した。主アッセイには、逆転写ポリメラーゼ連鎖反応法（RT-PCR）を用い、その結果はPCRベースの制限酵素分析で確認した（補遺の方法の項を参照）。

結　　果
調べた材料と不適当な検体の除外
合計40,022件のパラフィンワックスブロックを801個の容器に収めた：20,041件の検体はUCL Institute of Neurologyで、19,981件はAnimal Health and Veterinary Laboratoies Agencyで行われた。総合では、80,044個の免疫標識スライドについて最初の評価を行った。さらに、

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5,720件の対照の切片、および約4,000件（5%）のリピート試験切片を評価した。収集した40,022件の虫垂検体から19%（7,500検体）を除外したが、それは生きた濾胞が認められなかった、または濾胞が炎症によって圧倒されていたかもしくは破壊されていた、濾胞の数が5つ未満であった、またはその組織が萎縮していたことによる。除外率は容器ごとに異なり、50件中0件（6個の容器）から、50中に29（1個の容器）までのものがあった。リピート用の切片は1,995件作成されたが、その作成理由の多くは、器械の失敗（54%）、ワックス除去が不完全（14%）、ペルオキシダーゼ-ジアミノベンチジンのデブリの残存（12%）であった（補遺の表3を参照）。

虫垂検体中の異常PrPの検出とPRNP コドン129遺伝子型の分布
本調査は2000年から2012年までの間に行われた手術時に得られた虫垂を対象とした。32,441件の対象とした虫垂検体のうち、16件の検体が陽性の判定基準に合致した。それらの16件の虫垂のうち9件から選んだ切片を図に示している（図3 A-I）。陽性を示した濾胞の数、病変を示した濾胞性樹状細胞領域の大きさ、および免疫標識の強度は16件の検体間で相違があったが（例えば、図2 U-Y;表1）、陽性の標識は常に二次濾胞の肺中心内の濾胞性樹状細胞に限局していた。別の2件の虫垂検体が、「疑わしい」との判定基準に合致していた（図3 K, M;表1）。濾胞中心に「非特異的」な抗体結合が見られたのは9件の検体で、それは虫垂炎の結果として虫垂の壊死性分解が起こっていたことによる（例えば、図3 J.L.N）。残りの検体は「陰性」と分類された。
陽性を示した16件の検体のうち、8件はPRNPコドン129がメチオニンのホモ接合であり、4件がメチオニン-バリンのヘテロ接合、残りの4件がバリンのホモ接合体であった（表1）。「疑わしい」とされた2件のうちの1件はメチオニン-バリンのヘテロ接合で、もう一方はバリンのホモ接合であった。16件の陽性を示した虫垂では横断的および長手方向の切片をとることが出来た（表1）。陽性の濾胞は一様に分布していたわけではなく、クロスセクション上ではいくつかの隣接する濾胞中に強い標識が不規則に分布しており、また横断的もしくは長手方向の異なるレベルでは陽性の濾胞がより少ないかまたは全く認められなかった。16件の陽性検体中5件では、調べた全ての切片において、見出された濾胞のうちの2%から20%が陽性であった（コドン129遺伝子型はそのうちの3件ではメチオニンのホモ接合、残り2件はメチオニン-バリンのヘテロ接合、およびバリンのホモ接合であった）；7件の検体では25%から60%が陽性で(メチオニンのホモ接合が3件、メチオニン-バリンのヘテロ接合が2件、バリンのホモ接合が2件)、4件の検体では75%から89%が陽性であった（メチオニンのホモ接合が2件、メチオニン-バリンのヘテロ接合が1件、バリンのホモ接合が1件）。このように、形態、染色強度、分布、および陽性を示した濾胞の比率（図3）は、コドン129遺伝子型とは無関係であった（表1）。

本研究で用いた抗体間の相違
高い強度の標識が見られたのは抗PrP抗体ICSM35を用いた場合が多く（図2 A, B）、抗PrP抗体KG9を用いた場合はすぐ隣接する切片上の同一の濾胞中での免疫反応性が弱く示されることが多く（図2, F, G）、このことは孤発性クロイツフェルト・ヤコブ病の脳切片で見られるものとは異なっており、孤発性CJDの場合には双方の抗体とも同一のシグナルを示す（図2 C, D, H, I）。陽性を示した虫垂検体について様々な回収方法、例えばマイクロウェーブやオートクレーブをかける時間を長くしたり、ギ酸での前処理などを行っても、KG9との免疫反応性は増大しなかった。初回の試験で陽性を示した虫垂でICSM35とKG9との相違が観察されたので、さらに我々はよく確立された診断用抗体である3F4と12F10を、陽性標識を示した切片全てに対して隣接している切片に用いたが、その場合でも免疫標識は多くの場合、弱いかもしくは検出し得なかった（例えば、図2 K, L, P, Q）。脳の切片に対してはこれら4種の抗体は強力なシグナルをもたらし（図2 C, H, M, R, およびD, I, N, S）、またvCJD症例から得たギ酸処理扁桃生検検体でも強いシグナルが得られた（図2 E, J, O, T）。

炎症となった虫垂内での濾胞性樹状細胞の分解
顆粒球の浸潤は全くないか非常に少なかった濾胞性樹状細胞ではCD21による強い免疫標識が虫垂中の全ての濾胞中に見られたのに対し（補遺の図1 A-Cを参照）、中等度に炎症の見られる虫垂ではCD21陽性濾胞性樹状細胞の数がより少なく（補遺の図1 D-Fを参照）、炎症が重度な虫垂では陽性細胞は見当たらず、そのような重度の炎症では濾胞は炎症細胞によって圧倒されていた（補遺の図1 G-Iを参照）。このことは、炎症のある虫垂ではPrP免疫標識濾胞性樹状細胞が検出されないので、調査検体から炎症のある虫垂を除外することが正当であることを示すものである。

生年で分けたコホート、性別、および地理的エリアによる異常PrP出現の相違
本研究で観察された異常PrPの出現頻度は、人口100万人あたり493例であった（表2）。1941年から1960年に生まれた人々では100万人あたり733例であり（95%信頼区間は100万人あたり269例～1596例）、1961年から1985年に生まれた人々での100万人あたり412例（100万人あたり198例～758例）と有意な差はなかった。これらの2つの生年別コホートをさらに9つの生年別コホート(1941-45、1946-50、その他)に分けると、1961-65コホートを別にすれば各コホートに少なくとも1例の陽性虫垂があった（表2）。陽性の虫垂のうち10例が男性から、6例が女性から採取されたものではあったが、この差は統計学的には有意ではなかった。イングランド中の41カ所の病院（補遺の表4を参照）から集められた検体を3つの大きな地理的エリア（北東部および北西部；南東海岸部、南西部、およびロンドン；ならびに東部と西部のミッドランド）にグループ分けしたが、異常プリオンの出現について明白な地理的相違はなかった。調査した虫垂の全てが2000年から2012年の間に行われた手術の際に集められたものであり、vCJDとなった177症例には以前に手術歴があったことが知られているので、既知のvCJD症例由来の陽性虫垂が入り込んだことはないと結論づけられる。

考　　察
ホルマリンで固定しパラフィン中に包埋された虫垂32,441検体のうち、16件の検体が異常プリオンタンパク質（PrP）に対して陽性を示し、そのことは総合的には人口100万人あたり493例の出現があることを示していて、この出現率は性別および検体の得られた3つの広い地理的エリアにかかわらず同様であった。総合的な推定出現率である人口100万人あたり493例（95%信頼区間は100万人あたり282例～801例）は、以前の調査での推定（100万人あたり237例、49例～692例）の2倍となっているとはいえ、今回の推定出現範囲は初回調査のものと大きくオーバーラップしており、推定幅はより狭く、推定の中央値はより高くなっている（1:2000 vs. 1:4000）。今回の調査では生年別コホートで見た場合も、以前に調べたときに比べPrPの存在がより広い生年別コホートに広がっていることが示されている。

陽性を示した虫垂検体の遺伝子型
初回の虫垂調査の時の驚くべき知見は3件の陽性検体のうちの2件のPRNPコドン129遺伝子型が、バリンのホモ接合だったことであった。今回のより大規模な研究のほうが、陽性の虫垂での遺伝子型の頻度と免疫標識の相違をよりよく代表している：16件の陽性検体のうちの50%がPRNPコドン129の位置でメチオニンホモ接合、25%がメチオニン-バリンのヘテロ接合、25%がバリンのホモ接合であったが、これはこの人口集団での正常な分布であるそれぞれ43%、45%、および13%とは異なっている（補遺の図2を参照）。虫垂に関する2回の調査での知見をまとめると、これらの調査で遺伝子型の調べられた虫垂ではバリンのホモ接合出現頻度（18検体中6検体）は、英国の人口全体での遺伝子型出現頻度と比べて予測よりも高かった（P=0.02, Fisherの正確確率検定）。陽性を示した虫垂でバリンホモ接合遺伝子型がこのように増加していたことは、vCJD患者177例での結果とは異なっており、vCJD患者では現在までのところ全員がメチオニンホモ接合である（$P<10^{-5}$, Fisherの正確確率検定、補遺の図2を参照）。

抗体の技術的側面
技術的側面としては、今回の我々の研究では用いた抗体間の性能の差が明らかとなり、ICSM35は信号対雑音比がKG9、12F10、3F4よりも高かった。このことは3F4およびKG9が用いられていた以前の調査とは異なっている。以前の虫垂の調査では3F4とKG9が用いられていたがICSM35は用いられていなかった。用いた抗体は全て、生検材料（小脳）で皮質に弱いPrP標識を検出し（図2 D, I, N, S）、それらはギ酸処理した扁桃生検検体でヴァリデートされた（図2 E, J, O, T）。虫垂生検検体は手術時に得られたものであり、通常は迅速かつ短時間でホルマリン固定され、ギ酸では処理されない。ICSM35がそのような生検検体での異常PrP検出ではより感度が高い可能性があり、また他の3種類の抗体は、図1のE, J, O, Tで示唆されているように、ホルマリン固定後にギ酸処理を行ったために好結果が得られているのかもしれない。重要なことは、4種類の抗体全てが、陽性虫垂検体の免疫標識された濾胞中の同一の細胞タイプを検出したことである。いくつかの研究では、適切な回収技法と組み合わせた免疫組織化学的試験を異常PrP検出のためには十分な方法としてヴァリデートしており、異常PrPに特異的でホルマリン固定パラフィン包埋材料に適した抗体は得られていないにもかかわらず、保管していた材料中の異常PrPを検出するという前回の研究と今回の研究の有効性と信頼性に根拠を与えている。重要なのは、それらの研究でも異常PrPのリンパ網内系への蓄積がヒトの各種プリオン病の中でvCJDの持つ特徴であると結論づけていることである。

試験の感度
いくつかの理由で、免疫組織化学的に検出された虫垂の陽性は異常PrP出現を過小評価しているものかもしれない。第1に、虫垂ブロック中の組織はスクリーニングされた2つの隣接する切片によって十分に代表されているものとされている。1つまたは2～3の濾胞中に陽性の濾胞性樹状細胞を含んでいる場合にのみ、アンダーサンプリングが問題となるとはいえ、そのような生物学的な変動は我々の方法の感度を下げ、異常PrP出現を過小評価してしまう可能性がある。動物実験での発症機序の研究では、潜伏期間の早期では陽性の濾胞数は少なく、潜伏期間の経過とともに増加することが示唆されている。第2に、vCJD症例の死後行った検査での虫垂の陽性率は、20検体中19検体(95%)から4検体中1検体(25%)までばらつくことが見出されている（補遺の表1を参照）。第3に、我々はCD21免疫染色を用いて炎症が濾胞性樹状細胞を破壊し、それによって陽性となるはずであった検体の数を減少させることを確認した（図1）。しかし、我々の行った一連の試験では、炎症のために除外されたのは虫垂検体の12%にすぎず（補遺の表2を参照）、また調べた虫垂検体のうち急性炎症状態の虫垂のいずれにおいても異常PrPの標識は見られず、炎症がPrP沈着引き起こすことはないものと思われる。第4に、初回調査の時と同様に、今回の2回目の調査で、無症候性感染の発生を推定することおよびvCJDの将来的な発生数の予想をすることに限界があるのは、潜伏期間のどのステージで異常PrPがリンパ組織中に検出されうるようになるのかが不明なためである。

異常PrP出現の推定値とvCJD発生症例数との間に差があること
現在までのところ、暴露された人口集団内で観察されるvCJDプリオンの出現率とvCJDを発症した患者数が比較的少ないこととの間の差は拡大しつつあり、それは虫垂でのプリオン特異的免疫組織化学的試験の真の感度と特異性の如何によらず拡大している。臨床症状のあるvCJDの患者数（2013年6月時点で177例）は、異常プリオンの出現数から示唆される数をはるかに下回っており、vCJD患者数は異常プリオンも出現した人数のうちPRNPコドン129がメチオニンホモ接合である人数（6000例と推定される）をもはるかに下回っている。それでもやはり、それらのデータは最近の動物実験の結果に反してはおらず、それらの実験ではウシ海綿状脳症（BSE）についてのヒトの伝播バリアはおそらく臨床症状の発現する疾患としては高いが、末梢のリンパ網内系感染については相当低い可能性が示唆されている。臨床症状の発現する疾患（vCJDプリオンの中枢神経系への感染）にはメチオニンホモ接合の遺伝子型が非常になりやすいのであろうが、末梢リンパ網内系への感染はおそらく選択性がはるかに低いか、またはバリンのホモ接合遺伝子型の方がなりやすいということすらありうると思われる（補遺の図2を参照）。従って、この出現率のデータはいくつかの重要な問題点を提起している。第1に、メチオニン-バリンヘテロ接合もしくはバリンのホモ接合である宿主であって、免疫学的に陽性のリンパ網内系組織を有している宿主がvCJDの発症に対して防護されているのか否か、またはそのような宿主はやがては臨床症状のあるプリオン病を発症するのか、もしそうであるとするならば潜伏期間はどの程度長くなるのか、という点については不明である。第2に、メチオニンホモ接合の人がvCJDを発症する危険性が暴露時の年齢でどの程度低下するのか、またこのような低下が非常に大きく持続的に無症状のキャリアー状態がもたらされるのかは不明である。第3に、キャリアーがこの疾患を、手術手技を介して、もしくは血液やその他の組織のドネーションを介して伝播させる危険性を有しているのか否かは不明である。最後に、メチオニン-バリンヘテロ接合およびバリンホモ結合であるキャリアーが、臨床症状のあるプリオン病を発症するとタイプ4（タイプ2bとも言われる）にあたるPrP糖鎖型を伴うvCJDの臨床徴候を示すこととなるか否かは不明である。PrP糖鎖型は生化学的署名と言えるものであり、PrP分子の特定の部位に糖鎖付加されていることによって決定され、糖鎖型は孤発性CJDと変異型CJDを識別するものである。メチオニン-バリンヘテロ接合である人に、汚染された血液を介してvCJDが伝播して、vCJDに特徴的なタイプ4（2b）糖鎖型がもたらされる可能性もあり（メチオニンホモ接合の人と同様に）、また、いくつかの実験的伝播研究で示唆されているように、そのような糖鎖型の代わりに、新規のこれまでとは異なる表現型と糖鎖型を示す可能性もある。いくつかの答えは、症例の調査、特にPRNPコドン129遺伝子型のルーティンの検査と組み合わせた調査によって、またプリオン病（臨床症状が出ているか否かにかかわらず）の患者の生検で得られる中枢神経系検体のPrP糖鎖型の試験によって得られるかもしれない。ヒツジで行われた輸血に関する研究で得られたデータは、血液の感染性が潜伏期間の早期から存在しており、それは感染の主ルートが何であったかに関わらないことが示唆されている。もしも予防手段が遵守され、ヒトではリンパ網内系内に異常PrPが存在することは血液に感染性があることと同義であるという仮定が正しいとするならば、ここで観察された異常プリオンの出現の多さからみて、輸血によるvCJDの伝播が英国でこれまでにもっと多く見られてよいはずである。もしも輸血という経路での伝播がヒトで効率的に起こるとするならば、いくつかの仮定をおけば、数学的モデルからは、最初の感染を介してメチオニン-バリンのヘテロ接合およびバリンのホモ接合遺伝子型を有することとなった人々のどちらからも将来的にvCJDとなる人はごく少数であろうと考えられることが示唆され

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る。その代わり、全ての遺伝子型で二次的な医原性伝播の結果としてより多い数の症例が将来的に発生する可能性があり、そのような二次的な流行が起こり始めると数十年にわたってそのようになるであろう。ヒトにおけるBSE関連異常PrPの臨床経過がヒツジとは異なるに違いないとの結論を出す前に、ヒト血液中の異常PrPの出現率を測定することが賢明であろう。ヒト血液のスクリーニング試験法として満足できるものが大規模に行いうる形で登場した場合には、直ちにこのような個人と検体を直接結びつけない匿名化した調査を行うべきである。一方、虫垂での異常PrP出現率と輸血の結果として起こるvCJD症例数との間に差があることは輸血によるvCJDの伝播危険性が低いことを示唆はするが、血液のレシピエントのうちのどのくらいの数の人々が不顕性の疾患を有しており、それらの人々の寿命が潜伏期間よりも短いのか否かは不明である。従って、血液中の異常PrPを検出する試験法について研究を続けることは必須である。

結　　論
第2回目の虫垂の調査の結果、異常プリオン出現率について現在までで最もしっかりした尺度が得られ、その結果では、生年別コホートで見た場合、出現は広範であり、全ての遺伝子型でその出現が見られた。これらの知見の解釈は、BSEが動物間流行となる前の1970年代半ばから後半に手術で摘出された虫垂検体に関しての今後の調査、それは既に開始されているが、その調査の助けとなり、食物からの暴露のない場合の出現率に関する情報が得られるであろう。

報告企業の意見	今後の対応
血漿分画製剤は理論的な vCJD 伝播リスクを完全には排除できないため、投与の際には患者への説明が必要である旨を2003 年 5 月から添付文書に記載している。2009 年 2 月 17 日、英国健康保護庁(HPA)は vCJD に感染した供血者の血漿が含まれる原料から製造された第Ⅷ因子製剤の投与経験のある血友病患者一名から、vCJD 異常プリオン蛋白が検出されたと発表したが、原料血漿採取国である米国では、欧州滞在歴のある献（供）血希望者を一定の基準で除外している。また、国際獣疫事務局（OIE）において、米国は我が国と同じく「無視できる BSE リスク（Negligible BSE risk）」の国に認定されていることからも、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。なお、本剤の製造工程においてプリオンが低減される可能性を検討するための実験を継続して進めているところである。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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RESEARCH

Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: large scale survey

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Abstract

Objectives To carry out a further survey of archived appendix samples to understand better the differences between existing estimates of the prevalence of subclinical infection with prions after the bovine spongiform encephalopathy epizootic and to see whether a broader birth cohort was affected, and to understand better the implications for the management of blood and blood products and for the handling of surgical instruments.

Design Irreversibly unlinked and anonymised large scale survey of archived appendix samples.

Setting Archived appendix samples from the pathology departments of 41 UK hospitals participating in the earlier survey, and additional hospitals in regions with lower levels of participation in that survey.

Sample 32 441 archived appendix samples fixed in formalin and embedded in paraffin and tested for the presence of abnormal prion protein (PrP).

Results Of the 32 441 appendix samples 16 were positive for abnormal PrP, indicating an overall prevalence of 493 per million population (95% confidence interval 282 to 801 per million). The prevalence in those born in 1941-60 (733 per million, 269 to 1596 per million) did not differ significantly from those born between 1961 and 1985 (412 per million, 198 to 758 per million) and was similar in both sexes and across the three broad geographical areas sampled. Genetic testing of the positive specimens for the genotype at *PRNP* codon 129 revealed a high proportion that were valine homozygous compared with the frequency in the normal population, and in stark contrast with confirmed clinical

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Supplementary information, tables, and figure captions
Description of procedure for processing blocks
Supplementary figure 1
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cases of vCJD, all of which were methionine homozygous at *PRNP* codon 129.

Conclusions This study corroborates previous studies and suggests a high prevalence of infection with abnormal PrP, indicating vCJD carrier status in the population compared with the 177 vCJD cases to date. These findings have important implications for the management of blood and blood products and for the handling of surgical instruments.

Introduction

Variant Creutzfeldt-Jakob disease (vCJD) in the United Kingdom and some other countries[1 2] was caused by an exposure of the population to bovine spongiform encephalopathy (BSE) prions. Large numbers of the UK population were exposed to BSE prions in the late 1980s and early 1990s through contaminated meat products in the food chain. vCJD differs from other forms of Creutzfeldt-Jakob disease in that abnormal prion protein (PrP) has been detected in tonsil biopsy tissues before death and throughout the lymphoreticular system and some other tissues after death.[3] Accumulation of abnormal PrP in follicular dendritic cells of lymphoreticular tissue is thought to precede invasion of the central nervous system, and previous observations[4-5] have confirmed the presence of abnormal PrP in archived surgical samples removed before the development of clinical symptoms. Although there have been only 177 cases of vCJD to date in the United Kingdom, it is likely that subclinical carrier states of infection with vCJD prions exist, particularly on crossing a species barrier after widespread low dose exposure.[6-9]

In the United Kingdom, four prevalence surveys of abnormal PrP have been reported to date[5 10-12] (see supplementary table 1). In the face of continued uncertainty over the results of these surveys, in 2008 the Spongiform Encephalopathy Advisory Committee (SEAC (replaced in 2011 by the TSE Risk Assessment Subgroup of the Advisory Committee on Dangerous Pathogens) advised a second immunohistochemistry survey of archived appendix samples to increase understanding of the differences between existing estimates of the prevalence of subclinical abnormal prion infection and to see whether a broader birth cohort was affected. This paper reports on the findings of that second survey.

Methods

Sample collection, anonymisation, and data handling

To screen at least 30 000 satisfactory appendix samples from the 1941-60 and the 1961-85 birth cohorts, the Health Protection Agency (since 1 April 2013 part of Public Health England) collected 40 000 samples that had been removed at operations between 2000 and 2012. The source appendix tissue, archived in standard histology wax blocks (formalin fixed, paraffin embedded) in 41 participating hospitals (see supplementary table 4), was sent to Public Health England. Many of the collaborating hospitals had participated in the earlier appendix and tonsil survey[5] by providing samples from appendectomies before 2000 and agreed to provide additional samples. To improve geographical representation of samples, we contacted additional hospitals in regions with lower levels of participation in the earlier survey, and collaboration was sought from the heads of the histopathology departments.

At Public Health England, we selected a single block per case and forwarded blocks in collections ("bins") of 50 source appendix samples to the two collaborating prion screening laboratories, at University College London Institute of Neurology and the Animal Health and Veterinary Laboratories Agency, where sectioning, staining, and expert microscopic examination was conducted. After quality assessment of the sectioning and staining, each bin of samples was returned to the laboratory at Public Health England. Before permission was given to the screening laboratories to begin examination we completed a robust, irreversible unlinking and anonymisation procedure (fig 1⇓). At the Public Health England coordinating laboratory, we created a survey database that included the survey number, sex of the case, five year birth cohort, and broad geographical area where the original appendicectomy hospital was sited (fig 1, table 1⇓ and supplementary table 4). We calculated exact binomial confidence intervals for the prevalence estimates and compared prevalence between subgroups using Fisher's exact test with a 5% significance level.

Preparation of sections and immunohistochemical detection of abnormal PrP

From each paraffin block we cut a primary set of three sections. We detected abnormal PrP using mouse monoclonal antiPrP antibodies KG9 on one section and ICSM35[10 12 13] on another (see supplementary methods), visualised using a peroxidase-diaminobenzidine detection kit (DAB Map Ventana Medical System; Roche, Burgess Hill, UK) (fig 2 A-D, F-I⇓). To investigate suspect cases and confirm positive ones, we repeated the staining on additional, subsequent sections and with antiPrP monoclonal antibodies (3F4 and 12F10, see supplementary methods) (fig 2 P-S). Both laboratories used identical equipment (Ventana Discovery XT; Roche, Burgess Hill, UK), reagents, and protocols. We also stained half of the samples with haematoxylin and eosin to assess the proportion affected by inflammation. We assessed each section set to ensure the presence of five or more follicles and whether the tissue contained appendix biopsy tissue or other tissue that may have been collected in error. In addition to the appendix samples, we occasionally identified non-appendix tissue containing lymphoid follicles and we included this tissue when it met the criteria. From any samples with possible follicular immunoreactivity, we prepared 12 additional 4 µm sections and three 10 µm paraffin rolls (fig 1). To determine how the presence of florid granulocytic (purulent) inflammation affected the detection of follicular dendritic cells in secondary follicles, appendixes that met the inclusion criteria (≥5 follicles present), in particular where follicles were partially overrun by granulocytes, we carried out CD21 immunostaining—an established marker for follicular dendritic cells[14]—on five samples each with minimal, moderate, or severe inflammation (see supplementary fig 1).

Expert examination

Once irreversibly anonymised, expert examination at UCL Institute of Neurology (SB) or the Animal Health and Veterinary Laboratories Agency (MMS/YS) categorised samples as either positive, suspect, non-specific, or negative. A positive sample showed immunolabelling of a characteristic follicular dendritic cell network within a germinative centre of a follicle and at least one follicle had to contain a small network of immunopositive follicular dendritic cells. The positive follicular dendritic cells had to be present either in consecutive sections in the same follicle, or in a different follicle on a deeper section. A suspect sample had either weak immunoreactivity in a follicle that did not correspond to the typical pattern of PrP positive follicular dendritic cell, or weak, equivocal reactive staining that was not reproducible on consecutive sections (fig 3 K, M⇓). Specimens classified as non-specific showed antibody binding in the follicle

centres—for example, in degraded tissue that did not show typical follicular dendritic cell morphology (fig 3 J, L, N). Negative samples showed no immunoreactivity; specific labelling of non follicular dendritic cell structures within follicles, such as macrophages; non-specific (background) labelling inside the follicles; or labelling of structures outside the follicles (see supplementary fig 1). We then referred all section sets of interest (positive and suspect immunolabelling) to other experts (JWI and DH) for two independent repeat examinations, including staining of spare sections at the National CJD Research and Surveillance Unit (fig 3 C). Finally, at three meetings the expert histopathologists reviewed each written report and slide set to arrive at a consensus opinion of the findings.

Determination of PRNP codon 129 genotype

We determined the codon 129 genotype of positive samples and a selection of others using allele discrimination with minor groove binding probes. For primary assay, we used a reverse transcription polymerase chain reaction, which was confirmed with a polymerase chain reaction based restriction endonuclease analysis (see supplementary methods).

Results

Material examined and exclusion of unsuitable specimens

A total of 40 022 paraffin wax blocks were processed in 801 bins; 20 041 at UCL Institute of Neurology and 19 981 at the Animal Health and Veterinary Laboratories Agency. Overall, 80 044 immunolabelled slides underwent initial assessment. In addition, 5720 control sections and approximately 4000 repeat sections (5%) were assessed. From the 40 022 appendix specimens collected, 19% (7500 samples) were rejected because either no viable follicles were present, the follicles were overrun or destroyed by inflammation, fewer than five follicles were present, or the tissue was atrophied. The rejection rate varied between the sample bins, from zero in 50 (six bins) to 29 in 50 (one bin). Altogether, 1995 repeat sections were made usually because of machine failures (54%), incomplete dewaxing (14%), or peroxidase-diaminobenzidine debris (12%) (see supplementary table 3).

Detection of abnormal PrP in appendix samples and distribution of PRNP codon 129 genotypes

The survey included appendixes from operations conducted between 2000 and 2012. Of the 32 441 appendixes included, 16 met the criteria for being positive; selected sections for nine of these 16 appendixes are shown (fig 3 A-I). The number of positive follicles, the size of the affected follicular dendritic cell area, and the strength of the immunolabelling, varied between these 16 samples (for example, fig 2 U-Y; table 1), and positive labelling was always confined to the follicular dendritic cells in germinative centres of the secondary follicles. Another two appendix samples met the criteria for being suspect (fig 3 K, M; table 1). Nine samples showed non-specific antibody binding in the follicle centre, owing to necrotic degradation of the appendix as a result of appendicitis (for example, fig 3 J, L, N). The remaining samples were classified as negative.

Of the 16 positive appendix samples, eight were methionine homozygous at *PRNP* codon 129, four methionine-valine heterozygous, and four valine homozygous (table 1). One of the two suspect appendixes was methionine-valine heterozygous and the other was valine homozygous. Transverse and longitudinal sections were available in the 16 positive appendixes (table 1). Positive follicles were not uniformly distributed but showed an irregular distribution with strong labelling in several adjacent follicles on one cross section (fig 2 U-Y), and fewer or no positive follicles on a different transverse or longitudinal level. Between 2% and 20% of follicles present, in all available sections, were positive in five of the 16 positive samples (the codon 129 genotype was methionine homozygous in three, and methionine-valine heterozygous and valine homozygous in the others); between 25% and 60% in seven samples (three methionine homozygous, two methionine-valine heterozygous, and two valine homozygous) and 75% to 89% in four samples (two methionine homozygous, one methionine-valine heterozygous, and one valine homozygous). Thus the morphology, staining intensity, distribution, and proportion of positive follicles (fig 3) were independent of the codon 129 genotype (table 1).

Difference between antibodies used in this study

Strong labelling was usually produced by antiPrP antibody ICSM35 (fig 2 A, B), whereas antiPrP antibody KG9 generally showed weaker immunoreactivity in the same follicles on immediately adjacent sections (fig 2 F, G), in contrast with sporadic Creutzfeldt-Jakob disease brain sections, where both antibodies showed identical signals (fig 2 C, D, H, I). Application of different retrieval methods for the positive appendix samples, such as longer microwaving or autoclaving, or formic acid pretreatment, did not increase KG9 immunoreactivity. Having observed the difference between ICSM35 and KG9 on the initial positive appendixes, we further applied well established diagnostic antibodies 3F4 and 12F10[15 16] to sections adjacent to any positively labelled sections, and again immunolabelling generally was weaker or undetectable (for example, fig 2 K, L, P, Q). All four antibodies yielded a strong signal on brain sections (fig 2 C, H, M, R and D, I, N, S) and formic acid treated tonsil biopsy samples from vCJD cases (fig 2 E, J, O, T).

Follicular dendritic cells degradation in inflamed appendixes

Robust CD21 immunolabelling of follicular dendritic cell was seen in all follicles in appendixes with no or minimal granulocyte infiltration (see supplementary fig 1 A-C), whereas fewer CD21 positive follicular dendritic cells were seen in moderately inflamed appendixes (see supplementary fig 1 D-F) and none in severely inflamed appendixes, where follicles were overrun by inflammatory cells (see supplementary fig 1 G-I). This indicates that the exclusion of inflamed appendixes from the survey samples was justified, as no PrP immunolabelled follicular dendritic cell would have been detectable.

Prevalence of abnormal PrP by birth cohort, sex, and geographical area

The observed prevalence of abnormal PrP was 493 per million population overall (table 2⇓). The prevalence of 733 per million (95% confidence interval 269 to 1596 per million) seen in those born in 1941-60 was not significantly different from the 412 per million (198 to 758 per million) seen in those born between 1961 and 1985. When these two birth cohorts were subdivided into nine birth cohorts (1941-45, 1946-50, etc), there was at least one positive appendix in each cohort apart from the 1961-65 cohort (table 2). Although 10 of the positive appendixes

had been collected from men and six from women, the difference was not statistically significant. When the samples collected from 41 hospitals throughout England (see supplementary table 4) were grouped into three broad geographical areas (north east and north west; south east coast, south west, and London; and East and West Midlands), there were no apparent geographical differences in abnormal prion prevalence. Because all the survey appendixes were collected from operations conducted between 2000 and 2012, and the previous operative history of the 177 vCJD cases in the United Kingdom is known, it can be concluded that none of the positive appendixes came from known vCJD cases.

Discussion

In 32 441 formalin fixed paraffin embedded appendix samples, we found 16 specimens positive for abnormal prion protein (PrP), indicating a prevalence of 493 per million population overall, and it was similar in both sexes and across the three broad geographical areas sampled.

Although the overall prevalence estimate, 493 per million (95% confidence interval 282 to 801 per million), was double the point estimate measured in the earlier survey of appendixes (237 per million, 49 to 692 per million),[5] the estimated prevalence range largely overlapped that from the first survey but was narrower with a higher central estimate (1:2000 *v* 1:4000). The current survey also shows the presence of PrP across a wider birth cohort than found previously.

Genotypes of positive appendix samples

A striking finding of the first appendix survey was the presence of the valine homozygous genotype at *PRNP* codon 129[17] in two of the three positive samples. The present, larger study gives a more representative picture of the frequency of genotypes and the variation of immunolabelling in these positive appendixes: 50% of the 16 positive samples were methionine homozygous at *PRNP* codon 129, 25% methionine-valine heterozygous, and 25% valine homozygous, in contrast with the normal distribution in the population of 43%, 45%, and 13%, respectively (see supplementary fig 2). When we combined the findings from both appendix studies, a higher number of valine homozygous cases were observed than would be expected (6 of 18) in the genotyped appendixes compared with the overall UK population genotype frequency[18] (P=0.02, Fisher's exact test). This increase of valine homozygous genotypes in positive appendix specimens differed from the 177 patients with vCJD, all of whom to date have been methionine homozygotes ($P<10^{-5}$, Fisher's exact test, see supplementary fig 2).[1 3 19 20]

Technical aspects of the antibodies

On a technical aspect, our study revealed a performance difference between the antibodies used, whereby ICSM35 had a higher signal to noise ratio than either KG9, 12F10, or 3F4. This is in contrast with previous studies where 3F4 and KG9 were used. The previous appendix survey[21] used 3F4 and KG9 but not ICSM35. All antibodies detected weak cortical PrP labelling in autopsy material (fig 2 D, I, N, S) and were validated with formic acid treated tonsil biopsies (fig 2 E, J, O, T). The appendix biopsy samples were material from surgery, usually rapidly and short term fixed in formalin and not treated with formic acid. It is possible that ICSM35 was more sensitive at detecting abnormal PrP in such biopsy samples and that the other three antibodies benefit from formic acid treatment after formalin fixation, as suggested in figure 1 E, J, O, T.

Importantly, all four antibodies detected the same cell type in immunolabelled follicles of positive appendix samples.

Several studies have validated immunohistochemistry in combination with appropriate retrieval techniques as an adequate tool to detect abnormal PrP and underpin the validity and reliability of previous studies and the present study to detect abnormal PrP in archival material,[22 23] despite the unavailability of antibodies specific for abnormal PrP and suitable for formalin fixed, paraffin embedded material. Importantly, these studies also concluded that lymphoreticular accumulation of abnormal PrP is a specific feature of vCJD in prion diseases in humans.

Sensitivity of the tests

For several reasons, immunohistochemically detected positivity in appendixes may underestimate the prevalence of abnormal PrP. Firstly, it is assumed that tissue in an appendix block is adequately represented by the two adjacent sections that were screened. Although under-sampling would be a problem only in those appendixes that contained positive follicular dendritic cells in one or a few follicles, such biological variation could cause our method to have reduced sensitivity and lead to an underestimate of the prevalence of abnormal PrP. Animal pathogenesis studies suggest that at early stages of the incubation period the number of positive follicles is low and increases with incubation time.[24] Secondly, the proportion of positive appendixes at post-mortem examination in vCJD cases has been found to vary from 19 of 20 (95%)[21] to one of four (25%)[25] (see supplementary table 1). Thirdly, we confirmed by using CD21 immunostaining for follicular dendritic cell that inflammation destroys these cells, hence reducing the number of potentially positive samples (fig 1). In our series, however, only 12% of appendixes were rejected owing to inflammation (see supplementary table 2) and the absence of labelling of abnormal PrP in any of the acutely inflamed appendixes that were examined makes it unlikely that inflammation triggers PrP deposition. Fourthly, as with the first appendix survey, a limitation of the second survey for estimating the prevalence of asymptomatic infection and predicting future numbers of vCJD cases is that it is not known at what stage during the incubation period abnormal PrP can be detected in lymphoid tissue.

Discrepancy between prevalence estimates and vCJD incidence

To date the discrepancy is growing between the prevalence of vCJD prions observed in the exposed population and the relatively small number of patients who have developed vCJD, whatever the true sensitivity and specificity of prion specific immunohistochemistry in appendixes. The number of patients with clinically manifest vCJD (177 cases at June 2013) is well below the number suggested by the prevalence of abnormal prion, even for those who are only methionine homozygous at *PRNP* codon 129 (an estimated 6000 cases). Nevertheless, these data are in keeping with recent animal experiments, which suggest that the human transmission barrier for bovine spongiform encephalopathy (BSE) may be high for clinical disease but substantially lower for peripheral lymphoreticular infection.[26] Although clinical disease (infection of the central nervous system with vCJD prions) may greatly favour the methionine homozygous genotype, peripheral lymphoreticular infection may be much less selective, or even favour the valine homozygous genotype (see supplementary fig 2). Therefore, the prevalence data raise several important issues. Firstly, it is not known whether hosts who are methionine-valine heterozygous or valine homozygous and carry immunopositive

lymphoreticular tissues are protected from developing vCJD or if they will eventually develop clinical prion disease, and if so how prolonged the incubation period would be. Secondly, it is unclear the extent to which the risk of developing vCJD in someone who is methionine homozygous decreases with age at exposure and whether this decrease is so great that a perpetual asymptomatic carrier state is the result. Thirdly, it is not known whether carriers pose a risk of transmitting the disease through surgical procedures[27] or through blood and other tissue donation. Finally, it is not known whether host carriers who are methionine-valine heterozygous and valine homozygous and develop clinical prion disease will present with clinical signs of vCJD with the PrP glycotype corresponding to type 4[2 28] (also designated type 2b[29]). The PrP glycotype is a biochemical signature, determined by the glycosylation of specific sites of the PrP molecule which distinguishes sporadic and variant CJD.[2] It is possible that the transmission of vCJD through contaminated blood into people who are methionine-valine heterozygous[29 30] also results in a type 4 (2b) glycotype characteristic for vCJD (like those with methionine homozygosity) or may it instead present with a different, novel phenotype and glycotype, as suggested by some experimental transmission studies.[31-33] Some answers may come through case surveillance, especially when combined with routine testing of the *PRNP* codon 129 genotype, and by testing the PrP glycotype of central nervous system samples in all autopsies of patients with prion disease, regardless of their clinical presentation.

Data from blood transmission studies in sheep suggest that blood infectivity is present early in the incubation period, whatever the primary route of infection.[34] If a precautionary approach is adhered to, and it is assumed that the presence in humans of abnormal PrP in lymphoreticular tissue is synonymous with blood infectivity, then the observed prevalence of abnormal prion suggests that more instances of vCJD transmission by blood transfusion should have been recognised in the United Kingdom by now.[35] Should transmission by way of blood transfusion prove efficient in humans, subject to certain assumptions mathematical modelling suggests that only a small number of future vCJD cases may be expected to arise in both the methionine-valine heterozygous and valine homozygous genotypes through primary transmission. Instead, a larger number of future cases may occur as a result of secondary iatrogenic transmission in all genotypes, and should this secondary epidemic arise, it would do so over decades.[27]

Before concluding that the clinical course of BSE related abnormal PrP in humans must differ from that in sheep, it would be prudent to measure the prevalence of abnormal PrP in human blood. As soon as a satisfactory human blood screening test becomes available in a scalable format, such an unlinked anonymous survey should be undertaken. Meanwhile, although the discrepancy between prevalence of abnormal PrP in appendixes and observed cases of vCJD as a result of blood transmission suggests that the risks of transmission of vCJD by blood transfusion are low, it is unclear how many blood recipients may have subclinical disease and if their life expectancy is shorter than the incubation time. Therefore it is essential to continue research into tests to detect abnormal PrP in blood.

Conclusions

The second appendix survey has provided the most robust measure of abnormal prion prevalence to date, and has shown a wider birth cohort and all genotypes to be affected. Interpretation of these findings will be aided by a further survey, already begun, of appendix specimens surgically removed before the BSE epizootic, in the mid to late 1970s, to inform about prevalence in the absence of dietary exposure.

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Contributors: ONG, SB, JL, NA, and SM conceived and designed the study. SB, YS, AR-L, CK, RD, LB, MS, PW, PB, DAH, JWI, JB, MP, and SM analysed and interpreted the data. SB and NG drafted the article and SB, NG, JI, DH, and SM revised it critically for important intellectual content. All authors, external and internal, had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis. NG and SB gave final approval of the version to be published and are the guarantors.

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Ethical approval: This study was approved by the multicentre research ethics committee (reference No 08/H0405/69). As the residual tissue specimens were screened using the unlinked anonymous technique and ethical approval was obtained, the consent of the patients from whom the tissues originated was not required.

Data sharing: A full dataset and technical appendix is available with open access in the supplementary material. This includes digital histology images that can be accessed on request from the corresponding author at s.brandner@ucl.ac.uk.

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What is already known on this topic

Widespread exposure of the UK population to bovine spongiform encephalopathy prions led to the emergence of variant Creutzfeldt-Jakob disease (vCJD)

Previous studies estimated the prevalence of vCJD carrier status in the UK population through screening archival appendectomy and tonsillectomy specimens, but results were conflicting

What this study adds

This further large scale survey of appendix tissue measured a high prevalence of abnormal prion protein, and abnormal prion protein was identified across a wider birth cohort than in previously

Genetic testing of positive appendix samples for the *PRNP* codon 129 genotype revealed a high proportion of valine homozygotes compared with the frequency in the normal population

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Tables

Table 1| Summary of appendix samples with positive, suspect, or non-specific immunolabelling for abnormal prion protein (PrP), by sex, geographical area, birth cohort, codon 129 genotype, and analysis of PrP positive follicles,* and a detailed breakdown of ICSM35 immunolabelling, and number of follicles with weak (+), intermediate (++), and strong (+++) positivity†

Demographic and area information					Analysis of PrP positive and negative follicles							
					IKG9+	ICSM35 positivity in follicles					No positive/all follicles (%)	Fig No
Case No, survey No	Sex	Geographical area, England	Birth cohort	Codon 129	+to+++	+	++	+++	Negative	Section		
Positive samples:												
1, 13119	M	North	1946-50	MM	2	4	—	6	5	TS1	10/28 (35)	3A
					0	—	—	—	4	TS2		
					0	—	—	—	9	TS3		
2, 14784	F	South and London	1956-60	VV	1	—	—	1	4	TS	2/10 (20)	3G
					1	—	1	—	4	TS2		
3, 15048	M	South and London	1971-75	MV	0	1	2	1	0	TS1	4/7 (57)	3D
					0	—	—	—	3	TS2		
					0	—	—	—	0	LS		
4, 16937	M	South and London	1976-80	MM	2	—	3	1	0	TS	4/5 (80)	3B
					0	—	—	—	1	LS		
5, 25173	F	North	1966-70	VV	0	3	—	2	0	TS1	5/11 (45)	3H
					0	—	—	—	3	TS2		
					0	—	—	—	3	LS		
6, 25612	F	North	1971-75	MV	0	—	1	1	6	TS1	2/11 (18)	3E
					0	—	—	—	3	TS2		
7, 28441	M	Midlands and east	1956-60	MV	0	—	3	—	0	TS1	25/28 (89)	2A, B, F, G, K, L, P, Q
					0	1	1	1	1	TS2		
					2	9	6	4	2	LS		
8, 31327	F	North	1966-70	VV	2	—	—	4	5	TS1	4/11 (36)	3I
					0	—	—	—	0	TS2		
					0	—	—	—	2	LS		
9, 32182	F	Midlands and east	1956-60	VV	0	—	2	—	1	TS1	12/15 (80)	3F, 2U-Y
					0	—	2	4	0	TS2		
					0	—	3	1	2	TS3		
10, 34039	M	Midlands and east	1981-85	MM	0	—	—	1	21	TS1	1/41 (2)	
					0	—	—	—	5	TS2		
					0	—	—	—	14	LS		
11, 36521	M	South and London	1966-70	MV	0	—	—	—	0	TS1	1/4 (25)	
					1	1	—	—	3	TS2		
					0	—	—	—	0	TS3		
12, 38647	F	South and London	1981-85	MM	0	1	—	—	2	TS1	6/50 (12)	3C
					0	2	—	—	2	TS2		
					1	—	1	2	40	LS		
13, 39047	M	South and London	1976-80	MM	0	—	—	—	8	TS1	6/16 (38)	
					0	—	1	—	1	TS2		
					0	—	—	5	1	TS3		
14, 39121	M	South and London	1976-80	MM	0	—	—	—	0	TS1	1/9 (11)	
					1	—	—	1	5	TS2		

Table 1 (continued)

Demographic and area information					Analysis of PrP positive and negative follicles							
					KG9†	ICSM35‡ positivity in follicles						
Case No, survey No	Sex	Geographical area, England	Birth cohort	Codon 129	+to ++	+	++	+++	Negative	Section	No positive all follicles (%)	Fig No
					0	—	—	—	3	LS		
15, 42181	M	South and London	1951-55	MM	0	3	—	—	1	TS1	3/4 (75)	
					0	—	—	—	0	TS2		
					0	—	—	—	0	TS3		
16, 42660	M	South and London	1941-45	MM	1	1	2	—	0	TS1	3/5 (60)	
					0	1	—	—	1	TS2		
					2	1	—	2	0	LS1		
					1	1	1	1	2	LS2		
Suspect samples:												
1, 18992	F	Midlands and east	1956–1960	VV	0	—	—	—	4	TS1	1/10 (10)	3M
					0	1	—	—	5	TS2		
					0	—	—	—	0	TS3		
2, 20050	U	South and London	1961–1965	MV	0	—	—	—	0	TS	1/16 (6)	3K
					0	1	—	—	16	LS		
Non-specific staining:												
1, 15368	M	South and London	1951–1955	MM	—	—	—	—	—	—	—	3J
2, 19878	U	South and London	1961–1965	MM	—	—	—	—	—	—	—	3L
3, 20174	U	South and London	1981–1985	—	—	—	—	—	—	—	—	—
4, 28791	F	South and London	1961–1965	VV	—	—	—	—	—	—	—	3N
6, 25655	F	North	1971–1975	MM	—	—	—	—	—	—	—	—
5, 31000	F	North	1956–1960	—	—	—	—	—	—	—	—	—
6, 31616	M	Midlands and east	1976–1980	—	—	—	—	—	—	—	—	—
7, 31753	M	Midlands and east	1961–1965	—	—	—	—	—	—	—	—	—
8, 34591	F	Midlands and east	1981–1985	—	—	—	—	—	—	—	—	—
9, 42713	F	South and London	1951–1955	—	—	—	—	—	—	—	—	—

MM=methionine homozygous; VV=valine homozygous; MV=methionine-valine heterozygous; U=unknown.

Most of the specimens showed an uneven distribution of positive follicles—that is, only in one of several sections within the specimen (for example, 13119, 15048, 16937, 25173, 25612, 31372). A few showed a homogenous distribution of positive follicles across all parts of the specimen (for example, 14784, 28441 or 32182). This distribution was independent of the intensity of staining of individual follicles.

*Number of follicles positive with KG9.

†Specimens contained transverse sections (TS) and longitudinal sections (LS) of tissue with variable numbers of positive and total follicles.

‡Mouse monoclonal antiPrP antibody KG9.

§Mouse monoclonal antiPrP antibody ICSM35.

Table 2| Prevalence of abnormal prion protein in appendix samples from operations conducted in England between 2000 and 2012 by area, sex of patient, and five year birth cohort

	North east and north west		South east coast, south west, and London		East Midlands and West Midlands		Total (including unknown sex)
Birth cohort	Female	Male	Female	Male	Female	Male	
1941–45	0/165	0/163	0/296	1/286	0/183	0/192	1/1420
1946–50	0/196	1/209	0/401	0/348	0/272	0/262	1/1918
1951–55	0/248	0/263	0/474	1/372	0/285	0/288	1/2124
1956–60	0/321	0/316	1/590	0/502	1/357	1/389	3/2719
1961–65	0/451	0/420	0/662	0/609	0/433	0/493	0/3438
1966–70	2/502	0/554	0/730	1/774	0/512	0/574	3/4090
1971–75	1/524	0/530	0/885	1/808	0/520	0/620	2/4408
1976–80	0/594	0/634	0/1035	3/1049	0/633	0/640	3/5184
1981–85	0/824	0/864	1/1551	0/1417	0/707	1/868	2/7140
Total	3/3825	1/3953	2/6624	7/6165	1/3902	2/4326	16/32 441

Figures

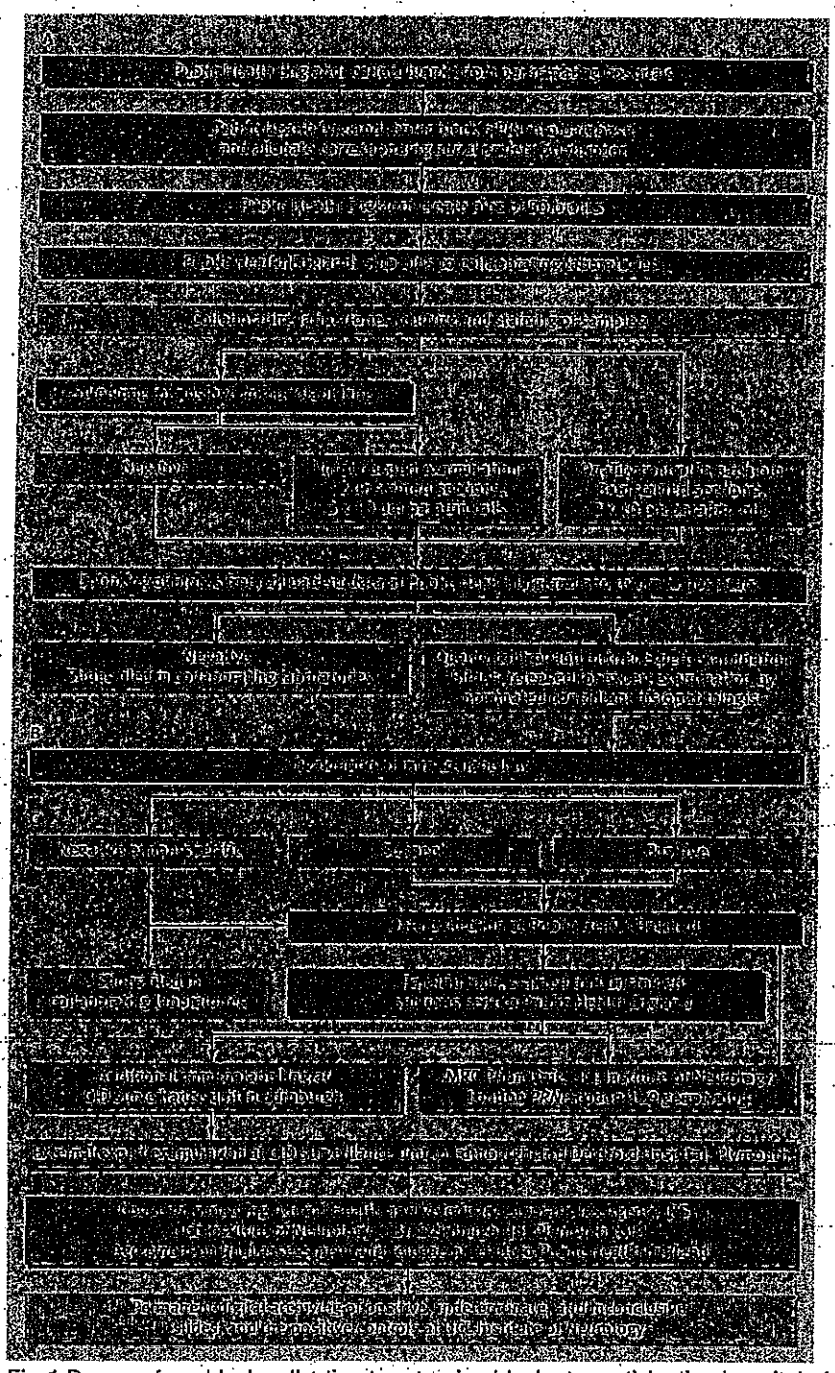

Fig 1 Process from block collection to returning blocks to participating hospitals (see supplementary file for full details)

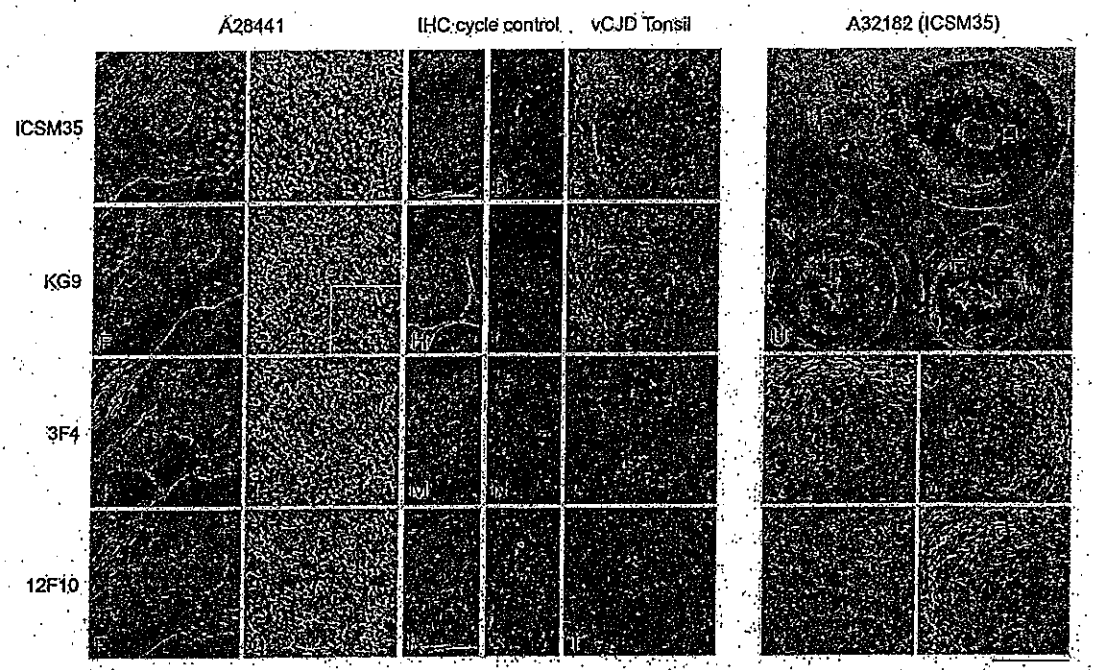

Fig 2 Immunolabelling of positive appendix samples and positive control sections stained during same machine run. First and second column: abnormal prion protein (PrP) in positive appendix sample (A28441). A, B: robust immunolabelling with antibody ICSM35 in several follicles (A). Higher magnification (B) shows characteristic labelling of follicular dendritic cells in one follicle. C, D: immunolabelling of section that served as external positive control in same machine cycle, containing section of cerebellum (C) or weak synaptic or perineuronal net labelling in the frontal cortex (D). F, G: antibody KG9 shows weaker but unequivocal immunolabelling of immediately adjacent section, whereas other antibodies (3F4, K, L) and 12F10, P, Q) do not show any signal. All cerebellar sections show robust immunolabelling (C, H, M, R) and there is less intense labelling of a cortical ribbon (D, I, N, S), characteristic of sporadic Creutzfeldt-Jakob disease with type 3 glycopattern. Antibody 12F10 shows slightly weaker detection of cortical PrP (N). Formic acid treated tonsil biopsy shows intense immunolabelling pattern with ICSM35 and KG9. U-Y: distribution of weak, intermediate, and strong immunolabelling of positive appendix (A32182). Red squares in low power overview (Q) correspond to panel R-U. Appendix shows positive labelling in 12 of 15 follicles. U; section corresponding to TS1 (see table 1) is on top, TS2 is located bottom left, and TS3 bottom right. Scale bar corresponds to 400 µm (first, third, and fourth columns; A, C, D, F, H, I, K, M, N, P, R, S), 100 µm (second column, B, G, L, Q), 6 mm (U), and 200 µm (vCJD tonsil column E, J, O, T and V-Y)

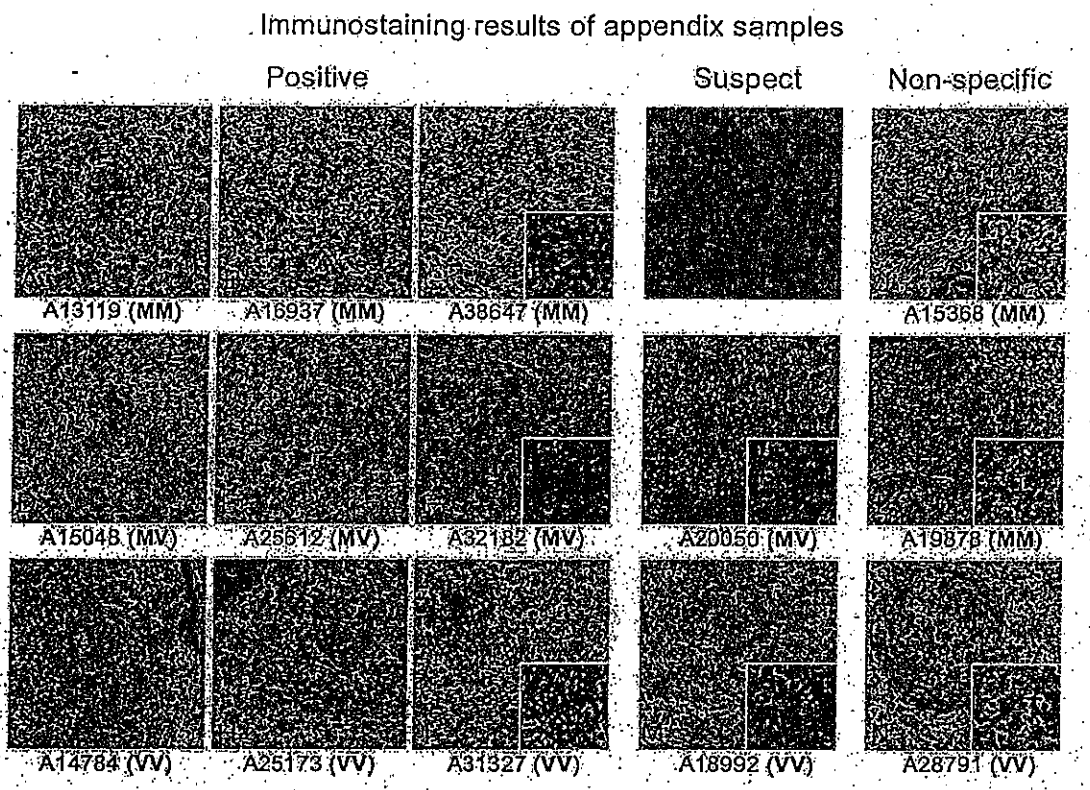

Fig 3 Examples of appendix samples diagnosed as positive, suspect, and non-specific for abnormal prion protein (PrP). A-C: examples of positive samples with methionine homozygous genotype at *PRNP* codon 129. Intrafollicular distribution and intensity of PrP show some variation, and all follicles show crisp, robust staining of follicular dendritic cells. Inset in C shows area with follicular dendritic cell at high magnification. D-F: three samples with variable positive labelling, illustrating variability in size and intensity of staining. Inset in F shows high magnification of follicular dendritic cell in centre. G-I: positive follicular dendritic cell in cases with valine homozygous genotype at *PRNP* codon 129, showing similar variability as previous cases. K, M: suspect cases, showing weak labelling of structures that may correspond to follicular dendritic cell, but not confirmed in subsequent immunostains. J, L, N: non-specific cases, with antibody binding to structures in follicle centre that do not correspond to viable follicular dendritic cell. J and N are necrotic follicles where follicular dendritic cell structure has disappeared, and L shows antibody binding in an area of poor morphological preservation. Scale bar 200 µm (50 µm in insets)

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識別番号・報告回数			報告日	第一報入手日 2013 年 11 月 25 日	新医薬品等の区分 該当なし	厚生労働省処理欄

一般的名称	人ハプトグロビン	研究報告の公表状況	www.ema.europa.eu/ema/index./ 2013/11/21	公表国 欧州
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研究報告の概要

尿由来医薬品における外来性物質の安全性に関するガイドライン（案）

1. はじめに（背景）

ヒトの尿は、ヒト絨毛膜性ゴナドトロピン（hCG）、ヒト閉経期ゴナドトロピン又はメノトロピン（HMG）および卵胞刺激ホルモン（FSH）および血栓溶解用に使われるウロキナーゼ製剤などの内分泌学の分野において示された幾つかの製剤を調製する（製造する）ために使われている。これらのホルモンおよびウロキナーゼはプールしたヒトの尿から抽出され、早くも 1970 年代に市販された。これらの製剤の製造プロセスの重要な改善は、より高い精度のプロファイルに到達するために 1990 年代に導入されている。並行して、販売承認申請資料はこの 10 年の間に設定されたウイルス安全基準に関して更新されている。尿は尿道内に宿るウイルスに自然に汚染される可能性がある。採尿時に生殖器や腸管から生じる他のウイルスは、尿のドネーションにおいて存在する可能性がある。製造プロセスのウイルスクリアランス能力の評価は、これらの医薬品の精製プロセスは外来性物質を除去/不活化することができる幾つかの工程を含んでいることを示している。これらのデータは、製剤のこの種のウイルス安全性記録が主に抽出および精製プロセスによるサポートを提供する。
1990 年代の変異型クロイツフェルト-ヤコブ病（vCJD ）の出現と UK におけるヒトの輸血による明らかな医原性 vCJD 感染の最近の症例は、EMA に CJD のこの新しい形に関する尿由来製剤の使用に関連するリスクを評価させた。専門家会合は 2002 年と 2007 年におけるこの質問に対応し、これらの評価の結果はクロイツフェルト-ヤコブ病および血漿由来医薬品および尿由来医薬品とその改訂に関する意見表明を含んだ（2003 年 2 月、2004 年 6 月および 2011 年 6 月）。

2. 範囲

ヒトの尿に由来した医薬品は以下のように指令書 2001/83/EC の第 1 条（2b）の定義に含まれている：「薬理学的な、免疫学的或いは代謝作用、或いは医学的診断を行うことにより、生理機能の回復、修正或いは変更するためにヒトで使われるかもしれない、或いは投薬されるかもしれない如何なる物質や物質の組み合わせ」。
このドキュメントは、ヒトの尿由来医薬品のウイルスおよび TSE 安全性の評価において考慮されるべき特定の側面に対応している。

3. 法的根拠

このガイドラインは、はじめにと一般原則（4）および改訂指図書 2001/83/EC の附属書 I を合わせて読むべきである。

4. 外来性物質の安全性

4.1. ドナーの選択

一般的に、尿のドネーションは、病気の場合にはドネーションを控えるように指示されたボランティアからである。その上、ドナーパネルの登録のために、メーカーはこれが可能である限り、一般的な健康状態に対する除外基準を設ける必要がある。従って、比較的小さなはっきり定義されたドナー集団から供給された尿由来ホルモンについては、製造業者はドナーの選択のための限られた除外基準を入れている。しかしながら、非常に大規模なドナープールから製造する他の製剤（例えば、ウロキナーゼ）については、その様な方法は適用が困難である。採尿は専門家の監督外で行われるので、血液/血漿ドナーとは異なり、各ドネーションでそれらの基準はチェック

使用上の注意記載状況・その他参考事項等

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

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されないであろう。このため製造業者は、定義された間隔でドナー基準をフォローアップする必要がある。

4.2. プロセス

2種類の原薬は、ヒトの尿－ホルモン（hCG、HMG、FSH）とウロキナーゼに由来している。製造戦略は、製品と製造業者により変わる。それらは一般的に保存剤の有無に係らず、個別採尿がプールされた後に適用される抽出、沈殿および精製工程からなる。尿は尿路に宿るウイルス、或いは生殖器や腸管に由来するウイルスで汚染されるかもしれない。出発原料として使用する大きな尿プールの検査に関連する制限事項を考慮に入れて、ウイルス安全性は主にウイルスを不活化、或いは除去するための製造プロセスの可能性に依存する。従って、製造業者は種々の物理化学的性質を示すウイルスを広範囲に不活化/除去するためにそれらの製造プロセスの能力を調査することが必要とされる。利用可能なデータは、製造プロセスの定義された工程で、尿プールを汚染する可能性があるウイルスの効率的なクリアランスを示唆する。より具体的には、ウロキナーゼ用の専用のウイルスクリアランス工程は、しばしば低温殺菌工程とナノろ過からなる。尿由来ホルモンに関しては、ウイルスクリアランスはアルカリ処理、沈殿、或いはクロマトグラフィー工程などの個々の製造プロセスに特異的なプロセス工程の組み合わせに起因している。尿由来ホルモンの製造業者は、高度に抵抗力のある、小さなノンエンベロープウイルスおよびウイルスろ過工程を含む幾つかの製造プロセスの更なる改良クリアランスのためにナノろ過を組み込むことが奨励されてきた。

出発原料が供給される場所の数のために、全ての採取システムのための場所での全体的な品質保証システムおよび製造プロセスの初期の製造工程のバリデーション/管理に対して、製造業者は特別な注意を払うべきである。

4.3. 製造プロセスの不活化/除去能力の調査研究

ウイルスの選択に関する一般的なガイダンスは、ウイルスバリデーション試験に関するガイダンスのための注意：ウイルスの不活化および除去を検証している試験のデザイン、寄与と解釈（CPMP/BWP/268/95、改訂）の中に定められている。このセクションは、尿由来品に関連した更なるガイダンスが含まれている。ヒトの尿中により頻繁に見つかるウイルスは、B型肝炎ウイルス（HBV）、ヒトサイトメガロウイルス（HCMV）、パピローマウイルスおよびポリオーマウイルス属のものである。糞便汚染によってもたらされる他のウイルスの存在は排除することができない（例えば、A型肝炎ウイルス（HAV）、或いは他のエンテロウイルス、E型肝炎ウイルス（HEV）、アデノウイルス、ノロウイルス、アストロウイルス、コロナウイルス様粒子、ロタウイルス）。

尿由来医薬品に関するバリデーション試験で使用されるウイルスには次のウイルスが含まれている必要がある：

エンベロープウイルス

・エンベロープRNAウイルス（例えば、ウシウイルス性下痢ウイルス（BVDV））

風疹ウイルス、ムンプスウイルスや麻疹ウイルスの様なエンベロープRNAウイルスは、急性感染の間にヒトの尿中に排出される。多数の更なるエンベロープウイルスからのRNAは、ヒトの尿中で検出されている。尿中の感染性エンベロープウイルス粒子の存在は多くの症例でありそうもないが、それは完全に排除することができない。種々のエンベロープRNA-モデルは、ウイルス不活化法を検証するために使われてきた。しかしながら、今日まで、ペスチウイルス ウシウイルス性下痢ウイルス（BVDV）は、他のRNAエンベロープウイルスのワースト・ケース・モデルと考えられる。

・エンベロープDNAウイルス（例えば、ヘルペスウイルス、仮性狂犬病ウイルス（PRV））

ヒトサイトメガロウイルス（HCMV）は、尿を介して伝播することができる。モデルDNAエンベロープウイルスへのパネルの中に仮性狂犬病ウイルス（PRV）の様なヘルペスウイルスが含まれることを推奨する。サイズ排除（ウイルスろ過）に基づく工程のバリデーションのために、ヘルペスウイルスを用いた試験は必要ない。現在、B型肝炎ウイルス力価測定のための実用的な検査システムはない。アヒルのB型肝炎ウイルス（DHBV）は、ヒトHBVのモデルとして使用することができる。しかし、力価測定のためにその自然の動物宿主（アヒル、或いは一次アヒル細胞）の使用を必要とする。その結果、ウイルスパネル中にDHBVを含める一般的な要件はない。

ノンエンベロープウイルスの SV40 および動物パルボウイルス

感染性ポリオーマウイルス、アデノウイルスおよびエンテロウイルスは、ヒトの尿中で見つけることができる。ポリオーマウイルス科のメンバーとしての SV40 は、バリデーション試験に使用すべきである。SV40 も、サイズ排除工程における HBV を表すために関連している。ヒトの糞便中の高力価で排泄することができるウイルスは、アデノウイルス、A 型肝炎ウイルス（HAV）および他のエンテロウイルス、E 型肝炎ウイルス、ノロウイルスおよびアストロウイルスの様な多くのノンエンベロープ DNA、或いは RNA ウイルスが含まれている。高度の耐性化した小型ノンエンベロープウイルスの適切なモデルは、パネルの中に組み込まれるべきである。これは、ブタ、イヌ、ウシおよびマウスパルボウイルスの中から選択することができる。幾つかの特異な症例では、モデルエンテロウイルス（例えば、1 つの工程がブタパルボウイルスの様なより抵抗性のウイルスに関して効果的であることを期待していない時）に対してパネルの中に HBV を含むことを正当化されるかもしれない。

4.4. 全体的なウイルスおよび TSE 安全

尿由来医薬品は、それらが感染性物質の伝播の原因であるかもしれないと疑われることなく、数年間多くの疾患の治療に使用されてきた。それでも、尿由来医薬品の全体的な伝播リスクについてのリスク評価を行うことは重要である。

以下は、それぞれの全体的なリスク分析の主要な構成要素である可能性が高い。それぞれの症例における分析の堅牢性の推定値は、有効に各構成要素を伴うかもしれない。

1. 採取が行われる地域、および特定のドナー集団（即ち、年齢、性別、および内分泌状態に基づいて）についてのウイルス疫学。
2. 疫学データに基づいて、そしてヒトの腎臓、尿路および生殖器管の病原体を宿す能力を考慮して、製品に関連する可能性が最も高い原因物質を同定することができるかもしれない。
3. 特定の集団の中でどの程度自分達が有効であるかという見積もりを含むドナーへの説明戦略、またドナーのモチベーション要因を含めたドナーの選択基準。
4. プールを開始するまでのドネーションおよび採取システム、およびセキュリティと衛生措置を含む。
5. 異なる製造業者/供給者の採取システムおよび早期の製造工程を管理するために製造者が従う品質保証システム、監査および手順に関する利用できるあらゆる情報。
6. 実施するスクリーニング検査を考慮したプール戦略。
7. 更に、適用される病原体のスクリーニング検査を考慮し、そして GMP 開始時点の特徴を含む抽出および精製方法論。
8. 適用される各々の TSE 原因物質およびウイルス除去工程の有効性、そして出発原料中で見つかるかもしれないウイルスとバリデーション試験で用いられるモデルウイルスでの結果との関連性。

実行可能であれば、病原体に汚染された尿由来医薬品の個々の用量の確率の推定値が企業によって考慮されなければならない。そのようなリスク分析は血漿由来医薬品用に開発された方法論に従うべきである、そして血漿由来医薬品ガイドラインおよび「クロイツフェルト・ヤコブ病および血漿由来医薬品と尿由来医薬品に関する CHMP のポジションステートメント」および「vCJD リスクに関する血漿由来医薬品の製造プロセスの調査」に関するガイドラインの中で論じた TSE 原因物質の低減に関するガイダンスにおいて記述されたウィルス安全面を考慮しなければならない。この様なリスク分析は、3.2.A.2 販売承認申請の偶然の原因物質安全性評価に表示すべきである。

医薬品
医薬部外品　研究報告　調査報告書
化粧品

報告企業の意見	今後の対応
血漿分画製剤は理論的なvCJD伝播リスクを完全には排除できないため、投与の際には患者への説明が必要である旨を2003年5月から添付文書に記載している。2009年2月17日、英国健康保護庁(HPA)はvCJDに感染した供血者の血漿が含まれる原料から製造された第Ⅷ因子製剤の投与経験のある血友病患者一名から、vCJD異常プリオン蛋白が検出されたと発表したが、日本血液製剤機構の原料血漿採取国である日本及び米国では、欧州滞在歴のある献（供）血希望者を一定の基準で除外している。また、第81回 国際獣疫事務局（OIE）総会（2013年5月）により、我が国が「無視できるBSEリスク」の国に認定されたことからも、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。なお、本剤の製造工程においてプリオンが低減される可能性を検討するための実験を継続して進めているところである。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

ハプトグロビン

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

1 London, 21 November 2013
2 EMA/CHMP/BWP/126802/2012
3 Committee for Medicinal Products for Human Use (CHMP)

4 Guideline on the adventitious agent safety of urine-5 derived medicinal products

6 Draft

Draft Agreed by Biologics Working Party	October 2013
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Date for coming into effect	

7
8 This guideline replaces the document on Biological products derived from human urine (CPMP/118/95).
9

Comments should be provided using this template. The completed comments form should be sent to alberto.ganan@ema.europa.eu

10

Keywords	Urine-derived medicinal products, adventitious agents, investigational studies

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Guideline on the adventitious agent safety of urine-derived medicinal products

Table of contents

1. Introduction (background)

Human urine is used to prepare several products indicated in the field of endocrinology, such as human chorionic gonadotropin (hCG), human menopausal gonadotropin or menotropin (HMG) and follicle-stimulating hormone (FSH) and urokinase products used for thrombolysis. These hormones and urokinase extracted from pooled human urine were available on the market as early as the 1970s. Significant improvements in the manufacturing processes of these products have been introduced in the 1990s in order to reach a higher purity profile. In parallel, marketing authorization dossiers have been updated as regards the viral safety standards set during this decade.[1] Urine may be naturally contaminated with viruses harboured in the urinary tract. Other viruses arising from the genital or intestinal tract during urine collection may be present in urine donation. Assessment of the viral clearance capacity of manufacturing processes has shown that the purification processes of these medicinal products contain several steps able to remove/inactivate adventitious agents. These data provide support that the viral safety record for this class of products is largely due to the extraction and purification processes.

The emergence of variant Creutzfeldt-Jakob disease (vCJD) in the 1990s and more recently the cases of apparent iatrogenic vCJD infection by blood transfusion in man in the UK prompted EMA to assess the risk linked to the use of urine-derived products as regards this new form of CJD. Expert meetings addressed this question in 2002 and 2007[2] and the results of these assessments were included in the Position statement on Creutzfeldt-Jakob disease and plasma-derived and urine-derived medicinal products and its revisions (February 2003, June 2004 and June 2011).[3]

2. Scope

Medicinal products derived from human urine fall under the definition of Article 1(2b) of Directive 2001/83/EC[4] as follows: "Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis."

This document addresses specific aspects which should be taken into consideration in the evaluation of viral and TSE safety of medicinal products derived from human urine.

3. Legal basis

This guideline should be read in conjunction with the introduction and general principles (4) and Annex I to Directive 2001/83/EC as amended.[4]

4. Adventitious agents safety

4.1. Selection of donors

Generally, donations of urine are from volunteers, who are instructed to refrain from donating in case of illness. In addition, for enrolment in a donor panel, manufacturers should establish exclusion criteria with respect to the general status of health as far as this is feasible. Accordingly, for urine-derived hormones, which are sourced from a relatively small well-defined donor population, manufacturers have put in place limited exclusion criteria for the selection of a donor. However, for other products manufactured from very large donor pools (e.g. urokinase), such measures are difficult to apply.

As urine collection takes place outside of professional supervision these criteria would not be checked at each donation unlike blood/plasma donors. Therefore manufacturers should follow up the donor criteria at defined intervals.

4.2. Processes

Two classes of drug substance are derived from human urine - hormones (hCG, hMG, FSH) and urokinase. Manufacturing strategies vary according to product and manufacturer. They generally consist of extraction, precipitation and purification steps, which are applied after individual urine collections, with or without preservative, have been pooled.

Urine may be contaminated with viruses harboured in the urinary tract or with viruses originating from the genital or intestinal tract. Taking into consideration limitations associated with testing of large urine pools used as starting material, virus safety mainly relies on the potential of the production process to inactivate or remove viruses. Manufacturers are therefore required to investigate the capacity of their manufacturing processes to inactivate/remove a broad range of viruses representing various physico-chemical properties. The available data suggest efficient clearance of viruses, which may contaminate the urine pool, by defined steps in the manufacturing process. More specifically, for urokinase dedicated viral clearance steps often consist of a pasteurisation step and nanofiltration. As regards the urine-derived hormones, virus clearance is attributed to a combination of process steps, which are specific for the individual manufacturing processes, such as alkali treatment, precipitation or chromatographic steps. Manufacturers of urinary-derived hormones have been encouraged to incorporate nanofiltration to further improve clearance of highly resistant, small non-enveloped viruses and several manufacturing processes include such a virus filtration step.

Due to the number of places where starting materials are sourced, particular attention should be given by manufacturers to the overall Quality Assurance System in place for the whole collection system and to the validation/control of the early production steps of the manufacturing process.[7]

4.3. Investigational studies of inactivation/reduction capacity of the manufacturing processes

General guidance on choice of viruses is given in the *Note for Guidance on virus validation studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses* (CPMP/BWP/268/95, revised).[1] This section contains further guidance relevant to urine derivatives. The viruses that are the more frequently found in human urine are hepatitis B virus (HBV), human cytomegalovirus (HCMV), and those from papillomavirus and polyomavirus genus. The presence of other viruses brought by faecal contamination cannot be excluded (e.g. hepatitis A virus (HAV) or other enteroviruses, hepatitis E virus (HEV), adenoviruses, noroviruses, astroviruses, coronavirus-like particles, rotaviruses).

Viruses to be used in validation studies on urine-derived medicinal products should include:

Enveloped viruses

- enveloped RNA viruses (e.g. bovine viral diarrhoea virus (BVDV))

Enveloped RNA viruses such as rubella virus, mumps virus or measles virus are shed into human urine during acute infection. RNA from numerous additional enveloped viruses has been detected in human urine. Even if the presence of infective enveloped virus particles in urine is unlikely in many cases, it cannot be totally excluded. Various enveloped RNA-models have been used to validate virus inactivation methods. However, to date, the pestivirus bovine viral diarrhoea virus (BVDV) is considered as a worst-case model for other RNA enveloped viruses.

- enveloped DNA viruses (e.g. herpesvirus, pseudorabies virus (PRV))

Human cytomegalovirus (HCMV) can be transmitted via urine. It is recommended to include a herpesvirus such as pseudorabies virus (PRV) in the panel to model DNA enveloped viruses. For the validation of steps based on size exclusion (virus filtration) studies with herpesviruses are not necessary. Currently, there is no practical test system for hepatitis B virus titration. The duck hepatitis B virus (DHBV) may be used as a model of human HBV. However, it requires the use of its natural animal host (duck or primary duck cells) for titration. In consequence, there is no general requirement to include DHBV in the virus panel.

Non-enveloped viruses SV40 and animal parvovirus

Infectious polyomaviruses, adenoviruses and enteroviruses can be found in human urine. SV40 as a member of the polyomaviridae virus family should be used in validation studies. SV40 is also relevant to represent HBV in size exclusion steps. Viruses which can be excreted at high titers in human stool include many non-enveloped DNA or RNA viruses such as adenoviruses, hepatitis A virus (HAV) and other enteroviruses, hepatitis E virus, noroviruses and astroviruses. An appropriate model for highly resistant small non-enveloped viruses should be incorporated in the panel. This may be chosen among porcine, canine, bovine and murine parvoviruses. In some specific cases, it may be justified to include HAV in the panel to model enteroviruses (for example when one step is not expected to be efficient on a more resistant virus like porcine parvovirus).

4.4. Overall viral and TSE safety

Urine-derived medicinal products have been used in the treatment of a number of conditions for several years without any suspicion that they are responsible for the transmission of any infectious agents. It is nevertheless fundamental to perform risk assessments for the overall transmission risks for urine-derived medicinal products.

The following are likely to be the main components of each overall risk analysis. Estimates of the robustness of the analysis in each case might usefully accompany each component.

1. Viral epidemiology for the region where collection takes place, and for the specific donor population there (i.e. on the basis of age, gender, and endocrinal status).
2. On the basis of the epidemiology data and taking into consideration the capacity for human kidneys, urinary and genital tracts to harbour pathogens, agents which are most likely to be relevant for the product could be identified.
3. Donor selection criteria, encompassing donor briefing strategies with an estimate of how effective they might be in particular populations, and donor motivation factors.
4. The donation and collection system up to the start of pooling, and including the security and hygienic measures in place.
5. Any information available on the Quality Assurance System, Audits and Procedures followed by the manufacturers to control the collection system and early production steps of the different manufacturers/suppliers.
6. Pooling strategies with a consideration of screening tests performed.
7. The extraction and purification methodologies, including a consideration of any further pathogen screening tests applied, and the indication of the point at which GMP starts.
8. The effectiveness of each TSE agent and virus elimination step applied and the relevance of the results obtained with model viruses used in validation studies with regards to the virus that may be found in the starting material.

Where practicable, consideration should be given by companies to presenting estimates of the probabilities of individual doses of a urine-derived medicinal product being contaminated with a pathogen. Such risk analyses should follow the methodologies developed for plasma derived medicinal

products and should take into account viral safety aspects described in the plasma derived medicinal products guideline[5] and the guidance concerning reduction of TSE agents discussed in the "CHMP Position statement on Creutzfeldt-Jakob Disease and Plasma-derived and Urine-derived Medicinal products"[3] and in the guideline on "Investigation of Manufacturing Processes for Plasma-derived Medicinal products with regard to vCJD risk".[6] Risk analyses of this nature should appear in 3.2.A.2 Adventitious Agent Safety Evaluation of Marketing Authorisation applications.

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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

○ブラジル中西部の供血者におけるリーシュマニア抗体

内臓リーシュマニア症(VL)は、世界で毎年20～40万人が罹患し、2～3万人が死亡する深刻な原虫疾患である。ブラジルでは、原虫*Leishmania infantum chagasi*がVLの原因であり、中南米で記録される症例の約90%がブラジルで発生している。

流行地域の供血者におけるリーシュマニア原虫の無症候性感染を調査するために、VL既往歴のない合計430人の供血者の血清検体における免疫蛍光抗体検査及び面談を実施した。調査の結果、67人(15.6%)にリーシュマニア抗体が検出され、感染と関連する因子として、「供血者の出身地」、「家の周囲に公園や広場がある」、「近所に皮膚疾患や四肢麻痺を呈する犬がいる」、「リーシュマニア症患者との接触」が挙げられた。また、多変量解析では、感染と出身地及び疾患を呈する犬との間に関連が見られた。

著者らは輸血の更なる安全性のため、リーシュマニア抗体のルーチン検査を行うべきであるとしている。

使用上の注意記載状況・その他参考事項等

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

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
ブラジル中西部の供血者においてリーシュマニア原虫の無症候性感染を調査するために血清検体の抗体検査を行ったところ、430人中67人(15.6%)が陽性であったとの報告である。	日本赤十字社では、輸血感染症対策として問診時に海外滞在歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、問診時にリーシュマニア症の既往歴を確認し、該当する場合は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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Anti-*Leishmania* antibodies in blood donors from the Midwest region of Brazil

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ABSTRACT

The aim of the study was to detect asymptomatic infection by *Leishmania* sp. in blood donors. Serum samples (430) were tested by Immunofluorescent Antibody Test, and an interview with the blood donors was carried out. Antibodies were detected in 15.6% of samples. The variables associated with the infection were: origin of the donor, presence of builds, parks or squares, sick dog in the neighborhood, and neighboring with leishmaniasis. It was observed association between origin of donors and the presence of sick dog. It is important a careful screening of donors, due to the risk of infection through blood transfusion.

1. Introduction

Visceral Leishmaniasis (VL) is a severe parasitic disease which affects between 200 and 400 thousand people worldwide, with annual incidence of approximately 20,000–30,000 deaths [1].

In Brazil, VL is a zoonosis caused by the protozoa *Leishmania infantum chagasi* and 90% of the all cases described in Latin America occur in this country. This disease is expanding geographically following urbanization processes and it is considered an emerging and reemerging disease in urban and rural areas [1]. VL is endemic in at least 21 Brazilian states, being considered a public health threat with outbreaks of difficult control [2,3]. In 2011 3894 cases of VL were confirmed, with a lethal index of 6.7%. In Mato Grosso do Sul this disease is reported in 56 out of 78 municipalities, with a high number of cases in Três Lagoas, Aquidauana and Corumbá. However, more than 50% of the cases were registered in the capital city of Campo Grande. In the last two years, an increase in confirmed cases (17%) was observed, but a reduction in the lethal index (42%) was observed as well [4].

Asymptomatic cases of VL have been reported in India [5], Iran [6], Italy [7], Iraq [8] and Brazil [9–13]. Based on this data, the risk of VL transmission by blood transfusion should not be ruled out. Considering several aspects such as severity of the disease, its evolution related to immunological status of the host, the immunosuppression of blood receptors, difficulties of control, epidemiological changes and the need to know aspects related to the transmission in different areas, more studies to evaluate the transmission risk of *Leishmania* sp. by blood transfusion are needed. Therefore, the aim of this study was to detect anti-*Leishmania* antibodies in blood donors in an endemic area in the Midwest of Brazil.

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2. Materials and methods

The study was carried out in the Hematology and Hemotherapy Institute of the state of Mato Grosso do Sul "*José Scaff*" – *Hemosul* of Campo Grande, MS, from April to November of 2011.

A total of 430 volunteers without history of infection by VL and clinically healthy were included in this study. All donors were submitted to an interview in order to obtain demographic, socioeconomic and epidemiological information. Patients who were seroreactive to *Trypanosoma cruzi* were excluded from the study. The present study received approval from the Ethics Committee in Human Research of the *Universidade Federal de Mato Grosso do Sul* (protocol nº1976 CAEE 0037.0.049.049-11).

Sera samples were evaluated by Immunofluorescent Antibody Test (IFAT) (Kit *Instituto Biomanguinhos da Fundação Instituto Osvaldo Cruz* – Rio de Janeiro), following the manufacturer's instructions. Samples were considered positive if IFAT titer ⩾1:80. Antigens utilized in this test were from *Leishmania major*-like obtained from cell culture. To perform this assay, the conjugate was titled and in each slide was included a negative and positive control. Slides were read by *two independent* laboratory technicians.

The statistical analyses were performed using Epi Info 3.5.3 (Centers for Diseases Control and Prevention, Atlanta/Georgia/USA) and BioEstat version 5.0 (*Sociedade Mamirauá*, Belém/Pará/Brazil). The chi-square test, chi-square of tendency and Fischer's test were used to verify associations among the variables studied and prevalence ratios, which were calculated with confidence intervals of 95%. To estimate the adjusted prevalence ratios, the Cox regression was utilized (with time equal to 1 unit), using in the preselection variables with significance <20%. The variables included in the final model were defined by the backward selection.

3. Results

Antibodies against *Leishmania* were detected in 15.6% (CI 95%: range from 12.2% to 19%) out of 430 blood donors. In the population studied 70.2% were male, median age was 32 years (range 18–68), 65.3% were locals from Campo Grande, 96.3% were living in this city and 79.1% were from the urban area.

Associations among *Leishmania* sp. infection and gender, age, school level, family income, animals, vegetation in peridomicile and/or neighborhood, presence of dogs, and knowledge about the diseases were not observed. Significant associations were observed only with the variables listed in Table 1.

The variables associated to the infection under adjusted analysis were: origin of the blood donor (Campo Grande versus other urban areas) and the presence of sick dog in the neighborhood with skin lesions and/or limb paresis (Table 2).

4. Discussion

The present study reports for the first time infection by *Leishmania* sp. in blood donors from the Midwest region of Brazil. Despite the difficulty in performing epidemiologic studies of this clinical form (asymptomatic infection), it is necessary to know its impact and importance in the epidemiology of parasites, in order to better understand the spread through transmission and the survival of the parasite in the carrier patient.

The seroprevalence of 15.6% found in this study was lower than those previously reported in the municipality of Três Lagoas/MS [11], where a prevalence of 36.4% among household members and contacts of carriers of VL was observed. However, the data from the present study confirms the presence of asymptomatic carriers of VL and serves as an alert to the possibility of these carriers acting as blood donors.

With respect to the group of blood donors, the prevalence observed in the present study was higher than that previously reported by Luz et al. [14] (seroprevalence of 9%) and Urias et al. [15] (5.5%) in Brazil, and in Spain (3.1%) and Italy (0.76%) [16,17]. However, in Greece and France similar prevalence were reported (15.2% and 13.4% respectively) [18,19].

The detection of antibodies against *Leishmania* reveals the induction of a humoral immune response, and is very useful in epidemiological surveys [20]. A positive reaction does not necessarily indicate active infection, but it may just be the result of a previous exposure to the parasite, especially in endemic regions [16,21]. This could explain the prevalence observed in this study, because the highest frequencies of reactive serum were observed in donors from the city of Campo Grande, MS, the main endemic area of the state, which during 2011 contributed with 57.14% of the cases reported in the state [4]. The origin of the blood donor remained associated to the infection after the multivariate analysis.

The presence of parks and squares around the homes of blood donors were associated to infection by *Leishmania* sp. This association may be justified by the fact that vegetation, often found in these locations, provide a suitable environment for the development of vectors of *L. infantum chagasi*, causing an increase in the incidence of the infection [22]. Another association observed (bivariate and multivariate analysis) in this study was between human infection and dogs with clinical signs of leishmaniasis (e.g., skin lesions, limb paresis). High susceptibility to *Leishmania* sp., intense cutaneous parasitism, as well as their close relationship with humans, makes the presence of seropositive dogs in human habitations a risk factor for infection by *L. infantum* [23].

The prevalence of infection was also higher among donors with a history of contact with people who died of leishmaniasis. Studies indicate that a history of VL on parents and neighbors is associated with higher rates of infection. Individuals living in the same residence with patients recently diagnosed with VL, have an increased risk for acquiring the disease of up to 26 times, confirming the role of these patients as reservoirs [11,24,25].

The risk of VL transmission by blood transfusion represents a major problem, due to the lack of a gold standard diagnostic method for the detection of asymptomatic infections. This risk has also been reported in Spain [16], Italy [17], France [19] and Brazil [15,26], with implications

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Table 1
Nonadjusted analysis of the association between infection with *Leishmania* sp.detected by the Immunofluorescence Antibody Test and demographic, socioeconomic and epidemiologic variables, Campo Grande, State of Mato Grosso do Sul, Brazil, 2013.

Variables	IFAT				PR crude (CI 95%)[a]	*p-Value*
	Positive (n = 67)		Negative (n = 363)			
	N°	%	N°	%		
Origin						
Campo Grande	54	19.2	227	80.8	1	0.004[b]
Country of MS/other	13	8.7	136	91.3	2.20 (1.24–3.90)	
Build/parks/squares						
Yes	7	30.4	16	69.6	1	0.050[c]
No	60	14.7	347	85.3	2.06 (1.07–4.00)	
Sick dog in the neighborhood with skin lesions/limb paresis						
Yes	5	41.7	7	58.3	1	0.012[b]
No	62	14.8	356	85.2	2.81 (1.38–5.70)	
Neighboring with leishmaniasis						
Death	5	29.4	12	70.6	1	0.050[b]
Treatment	6	8.0	69	92.0	3.68 (1.27–10.65)	
Absent	56	16.6	282	83.4	1.78 (0.82–3.85)	

Note: whether $p \leq 0.05$ – statistically significant difference.
[a] PR crude (CI 95%) = prevalence ratio crude with confidence intervals of 95%.
[b] Chi-square test.
[c] Fisher's exact test.

Table 2
Multivariate analysis of the association between infection with *Leishmania* sp.detected by the Immunofluorescence Antibody Test and demographic, socioeconomic and epidemiologic variables, Campo Grande, State of Mato Grosso do Sul, Brazil, 2013.

Variables	PR adjusted (IC 95%)	*p-Value*
Origin of donors (capital versus Country of MS/other)	2.18 (1.18–4.00)	0.012
Sick dog in the neighborhood with skin lesions/limb paresis	2.90 (1.16–7.25)	0.023
Nearby of build/parks/squares	2.01 (0.92–4.41)	0.081
Neighboring with leishmaniasis	1.76 (0.70–4.42)	0.226

on the role of the asymptomatic carrier as reservoir and their contribution to the maintenance of the transmission cycle of the parasite [5,27,28].

The use of leukodepletion filters seems to be the most efficient means to reduce risk of *Leishmania* transmission [16,29], improving quality and safety in the collection and transfusion of blood [30]. Among blood recipients, co-infections and immunosuppression may appear as aggravating factors triggering greater morbidity. In addition, the high cost of hospitalization for patients with VL, coupled with low availability of beds in public hospitals and toxicity of drugs used in the treatment should be considered.

There is a concern by the World Health Organization (WHO) to certify the safety of blood, and to make progress in relation to donor recruitment and blood collection, as well as in the routine testing on the samples of these donors [31]. The Pan American Health Organization [32] permanently refuses blood donations by individuals who had leishmaniasis, and does not allow donations, for a period of two years, of asymptomatic carriers or persons with a history of travel or transfusion at risk of being infected. The Council of Europe imposes the permanent refusal of potential donors with history of leishmaniasis. The Brazilian legislation on hemotherapy (DRC-ANVISA Resolution n°. 153/2004) recommends serology testing for Chagas disease, syphilis, HTLV 1 and 2, and HIV 1 and 2, as a routine in blood banks. Although this legislation considers those who have had VL permanently disabled for blood donation, there is no explicit recommendation to carrying out serology for *Leishmania* sp. [15].

In conclusion; routine diagnostic methods should be implemented in blood banks to exclude donors that are seropositive for*Leishmania* sp., promoting greater safety in blood bags available to the population. These measures can contribute to the quality of the blood supply and health of receivers, especially those with co-morbidities, as well as help expand on the knowledge about the epidemiology of VL and give support on improving measures to control the disease.

Authorship

All authors have made substantial contributions to the study design, analysis and manuscript.

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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

○米国カルフォルニア州サンディエゴ市の注射薬物使用者におけるE型肝炎ウイルス(HEV)、2009－2010年
注射薬物使用者におけるHEV感染の有病率データは限られている。
2009年3月～2010年6月、カルフォルニア州サンディエゴ市在住の、6カ月以内に注射薬物を使用した18～40歳を対象にHEV IgG抗体の血清陽性率及びそれに関連する要因を調査した。
その結果、508人の注射薬物使用者のうち14人(2.7%)がHEV IgG陽性で、HEV IgM及びRNA陽性者はいなかった。30歳以上の者は30歳未満の者より抗体陽性率が高かった(オッズ比3.61、95%CI 1.31-9.94)。旅行歴やHAV、HBV、HCV抗体の存在は、HEV抗体陽性とは関連しなかった。二変量ロジスティック回帰では、HEV IgG陽性と、受刑歴、注射器の共有、路上生活、高リスク性行動及びHIV感染の間に関連がないことが示された。

報告企業の意見	今後の対応
カルフォルニア州サンディエゴ市の注射薬物使用者において508人中14人(2.7%)がHEV IgG陽性であり、HEV抗体陽性率と他のリスク要因との間に関連はなかったとの報告である。	日本赤十字社では、厚生労働科学研究「経口感染によるウイルス性肝炎(A型及びE型)の感染防止、病態解明、遺伝的多様性及び治療に関する研究」班と共同して、献血者におけるHEV感染の疫学調査を行っている。今後もHEV感染の実態に関する情報の収集及び安全対策に努める。

使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-LR「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

Hepatitis E Virus among Persons Who Inject Drugs, San Diego, California, USA, 2009–2010

Reena Mahajan, Melissa G. Collier, Saleem Kamili, Jan Drobeniuc, Jazmine Cuevas-Mota, Richard S. Garfein, and Eyasu Teshale

Data about prevalence of hepatitis E virus infection in persons who inject drugs are limited. Among 18–40-year-old persons who inject drugs in California, USA, prevalence of antibodies against hepatitis E virus was 2.7%. This prevalence was associated with age but not with homelessness, incarceration, or high-risk sexual behavior.

Serologic evidence of hepatitis E virus (HEV) infection (i.e., IgG against HEV) in the United States has been reported to be ≤21% on the basis of national estimates during 1988–1994 (*1*). Among marginalized populations, such as persons who inject drugs (PWID) and homeless or incarcerated persons, HEV infection ranges from 5% to 23%, although data have been limited for these groups (*2–6*). We determined the seroprevalence of and factors associated with IgG against HEV among 18–40-year-old PWID in San Diego, California, USA.

The Study

Methods for the Study to Assess Hepatitis C Risk have been summarized (*7*). In brief, during March 2009–June 2010, persons 18–40 years of age who were residents of San Diego County, California, and who had injected drugs in the previous 6 months were recruited to participate in this study. Eligibility screening and acquisition of informed consent for potential participants were followed by a behavioral risk assessment and serologic testing.

Data collected included participant demographics, substance use, injection practices, diagnosis with sexually transmitted infections, exchange of sex for money, homelessness, travel to Mexico, and HIV status. Serologic testing included detection of antibodies against hepatitis A virus (HAV), hepatitis B virus core antigen, and hepatitis C virus (HCV) by using the VITROS Immunodiagnostic System (Ortho Clinical Diagnostics, Rochester, NY, USA), and IgG against HEV by using a commercial assay (DSI, Saronno, Italy).

We performed a comparative analysis of all persons on the basis of their status for IgG against HEV by using demographics, seropositivity for other viral hepatitides, travel to Mexico, history of incarceration, homelessness, HIV status, and high-risk sexual behavior. We used bivariate logistic regression to calculate odds ratios, 95% CIs, and p values, which were set at 0.05 to determine significance for factors associated with HEV prevalence. All data were analyzed by using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Of 508 PWID, 72% were men, their mean age was 29 years (range 18–40 years); and 62% were white. Fourteen (2.7%) persons had IgG against HEV; none of these persons were positive for HEV RNA by PCR (all were negative for IgM against HEV). Of the 14 persons with IgG against HEV, 11 (79%) were men; their mean age was 33.4 years (range 30–36 years); and 57% were white (Table). Relative to participants <30 years of age, persons ≥30 years of age were more likely to be positive for IgG against HEV (odds ratio 3.61, 95% CI 1.31–9.94). Travel history and presence of antibodies against HAV, hepatitis B virus, or HCV were not associated with presence of antibody against HEV. Bivariate logistic regression showed that there was no association between presence of IgG against HEV and a history of incarceration, sharing of injection drug equipment, homelessness, high-risk sexual behavior, and HIV status.

Conclusions

We found an overall HEV seroprevalence of 2.7% in young PWID in the United States. This seroprevalence was higher among participants ≥30 years of age than in participants <30 years of age. Variables typically associated with HCV/HIV transmission (i.e., high-risk sexual behavior, incarceration, or sharing of injection drug use equipment) were not associated with presence of antibodies against HEV. These findings were consistent with results of a study that found no association between antibodies against HEV and co-infection with other hepatitis viruses or sharing of drug paraphernalia (*2*).

Because of the common mode of fecal–oral transmission of HAV and HEV, other studies have also investigated an association between HAV and HEV infections, but results have been inconclusive (*1,5,6*). As in previous studies, we found an association of presence of antibodies against HEV and age (*1,6*). Higher prevalence among older PWID suggests that there may be age-related

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Table. Prevalence of IgG against hepatitis E virus among persons who inject drugs, San Diego, California, USA, 2010*

Characteristic	Positive for IgG against HEV, n = 14	Negative for antibody against HEV, n = 494†	p value
Sex			
M	11 (79)	357 (72)	0.707
F	3 (21)	137 (28)	ND
Mean age, y (95% CI)	33.4 (30.1–36.6)	28.5 (27.9–29.0)	<0.003
Age ≥30 y	9 (64)	170 (34)	0.013
Race			0.776
White	8 (57)	272 (55)	ND
Black	2 (14)	34 (7)	ND
Hispanic	2 (14)	137 (28)	ND
Other	1 (7)	15 (3)	ND
Homeless	11 (79)	287 (58)	0.326
History of incarceration	12 (86)	376 (76)	0.263
Sharing any drug injecting equipment	8 (57)	354 (72)	0.587
Diagnosis of sexually transmitted infection†	1 (7)	93 (19)	0.443
Exchange of sex for money	5 (36)	142 (29)	0.308
Travel to Mexico	10 (71)	314 (64)	0.373
Antibody against HAV	3 (21)	190 (38)	0.504
Antibody against HBc	3 (21)	201 (41)	0.138
Antibody against HCV	3 (21)	128 (26)	0.351
HIV positive	1 (7)	21 (4)	0.751

*Values are no. (%) unless otherwise indicated. ND, not determined; HAV, hepatitis A virus; HBc, hepatitis B core antigen; HCV, hepatitis C virus.
†Totals may not equal 100% because of missing data.

exposures independent of injection drug use that increases the likelihood of HEV infection. This birth cohort effect has been seen in other low-prevalence countries, such as Denmark (*8*), and decreased possible exposure may help explain the lower prevalence rates in our study.

This study had a few limitations. Our small sample size reduced the potential to detect significant differences between HEV-negative and HEV-positive persons. In addition, we did not have information about other exposures that have been associated with HEV infection, including particular dietary or zoonotic exposures or history of travel to a country to which HEV is endemic. Therefore, the potential effect of these exposures cannot be assessed. Information about HEV genotype was not available for seropositive persons, which might have provided clues as to the mechanism of exposure. Lower prevalence estimates may also reflect the fact that our population only included persons 18–40 years of age. Previous data have suggested that increasing age is associated with higher HEV positivity (*6*), particularly in countries in which prevalence is low and infection is caused mainly by HEV genotype 3 (*9*). Although our data cannot be generalized to the US population, seroprevalence in this study appears to be low, which is similar to time trends in the general population of other low-prevalence areas (*8*).

Variability in assay types used may account for discrepancies seen with previous seroprevalence studies of HEV. In a study evaluating the performance and concordance between various assays for detection of IgG against HEV available at the time, overall concordance ranged from 49% to 94% (median 69%), and concordance among reactive serum samples ranged from 0% to 89% (median 32%) (*10*).

Evaluation of the performance characteristics and concordance of currently available assays for detection of antibodies against HEV, including the assay used in this study, remains to be determined. Overall, our data showed an increase in antibodies against HEV for PWID ≥30 years of age and no other association with other reported risk factors. Future research is needed to explore other marginalized populations in HEV-endemic areas to determine whether there are other risk factors that have not been identified in low-prevalence areas.

Dr Mahajan is an Epidemic Intelligence Service officer at the Centers for Disease Control and Prevention, Atlanta, Georgia. Her research interests are national epidemiology, surveillance, and outbreak investigations related to viral hepatitis.

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医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日 2013年12月24日	新医薬品等の区分 該当なし。	総合機構処理欄
一　般　的　名　称	別紙のとおり。		研究報告の公表状況	WHO Influenza at the human-animal interface 2013; 20 December	公表国 中国	
販売名（企業名）	別紙のとおり。					

研究報告の概要	使用上の注意記載状況・その他参考事項等
問題点：トリインフルエンザA（H10N8）ウイルスの人感染例が初めてWHOへ報告された。 2013年12月17日、中国でトリインフルエンザA（H10N8）ウイルス感染のヒト死亡1例（女性 73歳，Jiangxi在住，基礎疾患のある免疫不全患者）が報告された。患者は73歳の基礎疾患を有する女性で、2013年11月30日に重症肺炎で入院し、12月6日に死亡しており、基礎疾患が重症化に寄与したものと思われる。患者は生きた家禽へ曝露歴があり、本症例はWHOへ報告された初めてのトリインフルエンザA（H10N8）の人感染例である。これまでに本患者との接触者から当該ウイルスは検出されていない。	記載なし。

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

MedDRA/J ver. 16.1

別紙

一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免役グロブリン、⑤人免役グロブリン、⑥乾燥ペプシン処理人免疫グロブリン、⑦乾燥スルホ化人免疫グロブリン、⑧乾燥スルホ化人免疫グロブリン、⑨乾燥スルホ化人免疫グロブリン、⑩乾燥スルホ化人免疫グロブリン、⑪乾燥スルホ化人免疫グロブリン*、⑫乾燥濃縮人活性化プロテインC、⑬乾燥濃縮人血液凝固第Ⅷ因子、⑭乾燥濃縮人血液凝固第Ⅷ因子、⑮乾燥濃縮人血液凝固第Ⅷ因子、⑯乾燥濃縮人血液凝固第Ⅸ因子、⑰乾燥濃縮人血液凝固第Ⅸ因子、⑱乾燥濃縮人血液凝固第Ⅸ因子、⑲乾燥抗破傷風人免疫グロブリン、⑳抗HBs人免疫グロブリン、㉑抗HBs人免疫グロブリン、㉒抗HBs人免疫グロブリン、㉓トロンビン、㉔フィブリノゲン加第ⅩⅢ因子*、㉕フィブリノゲン加第ⅩⅢ因子、㉖乾燥濃縮人アンチトロンビンⅢ、㉗ヒスタミン加人免疫グロブリン製剤、㉘人血清アルブミン*、㉙人血清アルブミン*、㉚乾燥ペプシン処理人免役グロブリン*、㉛乾燥濃縮人アンチトロンビンⅢ
販売名（企業名）	①献血アルブミン20“化血研”、②献血アルブミン25“化血研”、③人血清アルブミン“化血研”*、④ガンマーグロブリン筋注450mg/3mL「化血研」、⑤ガンマーグロブリン筋注1500mg/10mL「化血研」、⑥献血グロブリン注射用2500mg「化血研」、⑦献血ベニロンーⅠ静注用500mg、⑧献血ベニロンーⅠ静注用1000mg、⑨献血ベニロンーⅠ静注用2500mg、⑩献血ベニロンーⅠ静注用5000mg、⑪ベニロン*、⑫注射用アナクトC2,500単位、⑬コンファクトF注射用250、⑭コンファクトF注射用500、⑮コンファクトF注射用1000、⑯ノバクトM静注用400単位、⑰ノバクトM静注用800単位、⑱ノバクトM静注用1600単位、⑲テタノセーラ筋注用250単位、⑳ヘパトセーラ筋注200単位/mL、㉑ヘパトセーラ筋注200単位/1mL、㉒ヘパトセーラ筋注1000単位/5mL、㉓トロンビン“化血研”、㉔ボルヒール*、㉕ボルヒール組織接着用、㉖アンスロビンP500注射用、㉗ヒスダグロビン皮下注用、㉘アルブミン20%化血研*、㉙アルブミン5%化血研*、㉚静注グロブリン*、㉛アンスロビンP1500注射用
報告企業の意見	インフルエンザウイルスは70～120nmの球形または多形性で、核酸は8本の分節状マイナス一本鎖RNA、エンベロープを有し、エンベロープの表面に存在する赤血球凝集素(HA)とノイラミダーゼ(NA)の抗原性により16種類のHA亜型および9種類のNA亜型に分類される。 今回の報告は、WHOへ初めて鳥インフルエンザA（H10N8）の人感染例が報告された、というものである。 上記製剤の製造工程には、冷エタノール分画工程及びウイルス除去膜ろ過工程といった原理の異なるウイルスクリアランス工程が導入されており、各工程のウイルスクリアランス効果は「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（医薬発第1047号、平成11年8月30日）」に基づく、モデルウイルスを用いたウイルスプロセスバリデーションにより確認されている。今回報告した鳥インフルエンザA（H10N8）のモデルウイルスには、エンベロープの有無、核酸の種類等から、ウシウイルス性下痢ウイルス（BVDV）が該当すると考えられるが、上記工程のBVDVクリアランス効果については上記バリデーションにより確認されている。また、これまでに上記製剤による鳥インフルエンザA（H10N8）への感染報告例は無い。 以上の点から、上記製剤はインフルエンザに対する安全性を確保していると考える。

*：現在製造を行っていない

World Health Organization

Influenza at the human-animal interface

Summary and assessment as of 20 December 2013

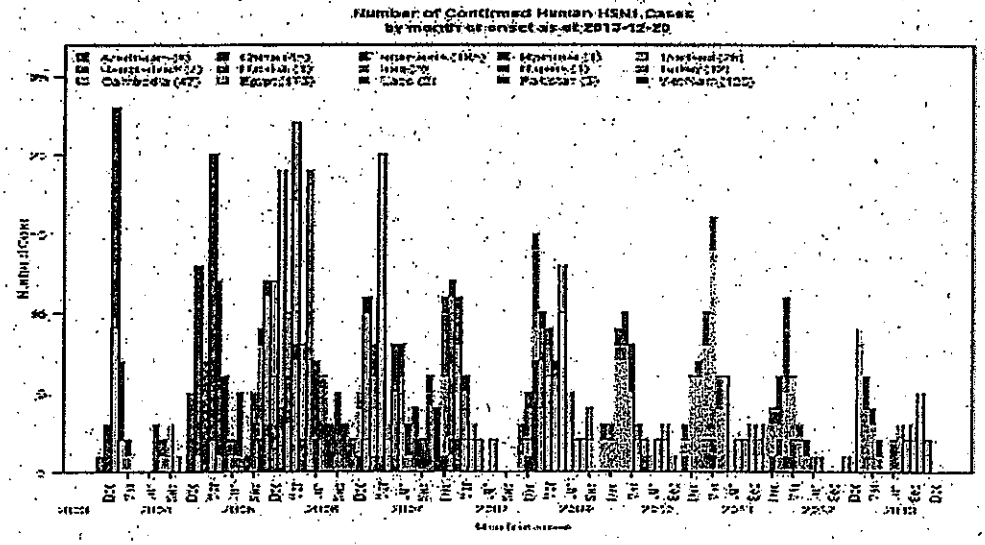
Human infection with avian influenza A(H5N1) viruses

From 2003 through 20 December 2013, 648 laboratory-confirmed human cases of avian influenza A(H5N1) virus infection have been officially reported to WHO from 15 countries. Of these cases, 384 died.

Since the last WHO Influenza at the Human-Animal Interface update on 10 December 2013, no new laboratory-confirmed human cases of influenza A(H5N1) virus infection were reported to WHO.

Overall public health risk assessment for avian influenza A(H5N1) viruses: Whenever influenza viruses are circulating in poultry, sporadic infections or small clusters of human cases are possible, especially in people exposed to infected household poultry or contaminated environments. However, this influenza A(H5N1) virus does not currently appear to transmit easily among people. As such, the risk of community-level spread of this virus remains low.

Figure 1: Epidemiological curve of avian influenza A(H5N1) cases in humans by country and month of onset



Human infection with other non-seasonal influenza viruses

Avian influenza A(H7N9) in China

Since the last update of 10 December 2013, China has reported four new cases of human infection with avian influenza A(H7N9) virus, from Guangdong province, with onset dates between 6 and 11 December. All new patients are in a critical condition.

Most human A(H7N9) cases have reported contact with poultry or live bird markets. Knowledge about the main virus reservoirs, and the extent and distribution of the virus in animals remains limited. Because this virus causes only subclinical infections in poultry, it is possible that the virus continues to circulate in China and perhaps in neighbouring countries without being detected. As such, reports of additional human cases and infections in animals would not be unexpected, especially with onset of winter in the Northern Hemisphere and as poultry production and movement increase in the region in anticipation of the Chinese New Year Holidays.

Although five small family clusters have been reported, evidence does not currently support sustained human-to-human transmission of this virus.

Overall public health risk assessment for avian influenza A(H7N9) virus: Sporadic human cases and small clusters would not be unexpected in previously affected areas of China, and possibly neighbouring countries. The current likelihood of community-level spread of this virus is considered to be low.

Continued vigilance is needed within China and neighbouring areas to detect infections in animals and humans. WHO advises countries to continue surveillance and other preparedness actions, including ensuring appropriate laboratory capacity. All human infections with non-seasonal influenza viruses such as avian influenza A(H7N9) are reportable to WHO under the International Health Regulations (IHR)2005.

Symptomatic individuals with exposure to avian influenza A(H7N9) virus should receive prompt antiviral *treatment*. Antiviral chemoprophylaxis post avian influenza A(H7N9) virus exposure is generally not recommended. For asymptomatic individuals at high-risk due to type of exposure or underlying conditions who have been exposed to a patient with confirmed avian influenza A(H7N9) virus infection, presumptive antiviral *treatment* with oral oseltamivir or inhaled zanamivir can be considered.

Current technical information as well as guidance related to avian influenza A(H7N9) can be found at: http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/index.html

Avian influenza A(H10N8) in China

On 17 December, China reported a human infection with avian influenza A(H10N8) virus in Jiangxi province. The patient was a 73 year-old female with multiple comorbidities, who was hospitalized on 30 November with severe pneumonia and died 6 December. The comorbidities of the patient might have contributed to a more severe illness than if the patient had been previously healthy. The patient had a history of exposure to a live bird market. Although avian influenza A(H10N8) virus has been previously reported in wild and domestic birds[1,2], this is the first human case of influenza A(H10N8) infection

[1] Zhang, H et al., Virology Journal, 2011

[2] Jiao, P et al, Journal of Virology, 2012

reported to WHO. No cases among contacts have so far been detected although follow up is continuing. Both health and agricultural authorities continue to enhance influenza surveillance in humans, poultry and the environment in the region, especially in the context of identifying avian influenza A(H7N9) virus-associated events.

Overall public health risk assessment for avian influenza A(H10N8)virus: Based on current epidemiological information this seems to be a sporadic human infection with avian influenza A(H10N8) virus, perhaps detected as a result of the current increased influenza surveillance in China. Although the prevalence of the virus in the local poultry population is unknown, sporadic cases of human infection with avian influenza A(H10N8) would not be unexpected if the virus were circulating in populations of birds to which humans were exposed.

Influenza A(H3N2) variant virus infections in humans in the USA

Since the last update of 10 December 2013, no new case of human infection with influenza A(H3N2)v was reported from United States of America (USA). To date in 2013, the USA has reported 19 cases of human infection with influenza A(H3N2)v.

Overall public health risk assessment for avian influenza A(H3N2)v virus: Further human cases and small clusters could occur as this virus is circulating in the swine population in the USA. However, agricultural fairs, where most of the human cases were reportedly exposed, are rarely held in the USA in the winter season. The current likelihood of community-level spread and public health impact of this virus is considered low.

Outbreaks in animals with highly pathogenic avian influenza viruses with potential public health impact

Owing in part to the emergence of avian influenza A(H7N9) virus and associated infections of humans in China, there is enhanced surveillance for non-seasonal subtypes of influenza in both humans and animals in China, the countries neighbouring China, and globally. It is therefore to be expected that more avian influenza A(H5N1), A(H7N9), and a variety of other influenza subtypes and reassortant viruses will be detected in humans and animals over the coming months . In order to detect virological and epidemiological changes that may affect public (or animal) health, it is critical that all non–seasonal influenza events be reported through the appropriate channels and that viruses be collected and fully characterized in appropriate animal or human health influenza reference laboratories.

Because of the constantly evolving nature of influenza viruses, WHO continues to stress the importance of global monitoring of influenza viruses in animals and people and recommends that all Member States strengthen routine influenza surveillance. All human infections with non-seasonal influenza viruses are reportable to WHO under the IHR (2005).

Links:

WHO human-animal interface web page
http://www.who.int/influenza/human_animal_interface/en/

Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO
http://www.who.int/influenza/human_animal_interface/EN_GIP_LatestCumulativeNumberH5N1cases.pdf

H5N1 avian influenza: timeline of major events
http://www.who.int/influenza/human_animal_interface/avian_influenza/H5N1_avian_influenza_update.pdf

Avian influenza A(H7N9) information
http://who.int/influenza/human_animal_interface/influenza_h7n9/en/index.html

World Organisation of Animal Health (OIE) web page: Web portal on Avian Influenza
http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/

Food and Agriculture Organization of the UN (FAO) webpage: Avian Influenza
http://www.fao.org/avianflu/en/index.html

OFFLU
http://www.offlu.net/index.html

別紙様式第２

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 2013年12月24日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	人C1-インアクチベーター		研究報告の公表状況	ProMed http://www.promedmail.org/direct.php?id=2121718	公表国 中国	
販売名（企業名）	①ベリナートP ②ベリナートP静注用 （CSLベーリング株式会社）					

研究報告の概要	香港衛生署（DH）衛生防護中心（CHP）は2013年12月17日、中国国家衛生家族計画委員会（NHFPC）から、鳥インフルエンザA（H10N8）ウイルスに感染した江西省の73歳の女性が死亡したとの報告を受けた。複数の基礎疾患があり易感染性状態であった同患者は、2013年11月30日に治療のため地元の病院に入院した。同患者の臨床診断は重度の肺炎で、2013年12月6日に死亡した。関係当局によると、同患者は地元の生鳥市場を訪れていた。同患者の自宅や密接な接触者は医学的監視の下に置かれているが、これまでのところ症候や異常は認められていない。 本件は新規のトリインフルエンザ（H10N8）ウイルス感染症例に関する報告である。

報告企業の意見	今後の対応
原料血漿の原産国は欧州および米国である。 製造工程にインフルエンザウイルスに有効な不活化/除去工程を有することから、インフルエンザウイルス（H10N8 型）に対する安全対策は取られていると考える。	今後とも新しい感染症に関する情報収集に努める所存である。

使用上の注意記載状況・その他参考事項等

Published Date: 2013-12-17 17:12:54
Subject: PRO/AH/EDR> Avian influenza, human (158): China (ZJ) H10N8, fatal
Archive Number: 20131217.2121718

AVIAN INFLUENZA, HUMAN (158): CHINA (ZIANGXI) H10N8, FATAL
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A ProMED-mail post
http://www.promedmail.org
ProMED-mail is a program of the
International Society for Infectious Diseases
http://www.isid.org

Date: Tue 17 Dec 2013
Source: Center for Health Protection (CHP), Hong Kong [edited]
<www.chp.gov.hk/en/view_content/24244.html>

Human fatal case of avian influenza A(H10N8) in Jiangxi province
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The [Hong Kong] Centre for Health Protection (CHP) of the Department of Health (DH) today (17 Dec 2013) received notification from the National Health and Family Planning Commission (NHFPC) of a human fatal case of avian influenza A(H10N8) affecting a woman aged 73 in Jiangxi. The immunocompromised patient with underlying illnesses was admitted to a local hospital on 30 Nov (2013) for treatment. Her clinical diagnosis was severe pneumonia and she passed away on 6 Dec (2013). According to the relevant authority, the patient had visited a local live poultry market. Her home and close contacts, who are under medical surveillance, have remained asymptomatic and no abnormalities have been found so far.

"Influenza A(H10) is currently not a local statutorily notifiable infectious disease but the Public Health Laboratory Services Branch of the CHP is capable of detecting this virus by culture or genetic testing. No confirmed human cases have been recorded so far in Hong Kong," a spokesman for the CHP said. The CHP will follow-up with the World Health Organization (WHO) and the mainland health authorities to obtain more information on the case.

"Locally, enhanced disease surveillance, port health measures and health education against avian influenza have been proceeding. We will remain vigilant and maintain liaison with the WHO and relevant health authorities. Local surveillance activities will be modified upon the WHO's recommendations," the spokesman remarked. All border control points have implemented disease prevention and control measures. Suspected cases of infectious disease will be immediately referred to public hospitals for follow-up investigation.

The spokesman urged travellers not to visit live poultry markets and avoid direct contact with poultry, birds and their droppings during travel. If contact has been made, they should thoroughly wash their hands with soap and water. If fever or respiratory symptoms develop, they should immediately wear facial masks, seek medical attention and reveal their travel history to doctors. Members of the public should remain vigilant and are reminded to take heed of the following preventive advice against avian influenza:

- poultry and eggs should be thoroughly cooked before eating;
- wash hands frequently with soap, especially before touching the mouth, nose or eyes, handling food or eating; after going to the toilet or touching public installations or equipment such as escalator handrails, elevator control panels or door knobs; or when hands are dirtied by respiratory secretions after coughing or sneezing;
- cover the nose and mouth while sneezing or coughing, and hold the spit with a tissue and put it into a covered dustbin;
- Avoid crowded places and contact with fever patients; and
- wear a mask when respiratory symptoms develop or when taking care of fever patients.

The public may visit the CHP's avian influenza page (www.chp.gov.hk/en/view_content/24244.html) and

its website (www.chp.gov.hk/files/pdf/global_statistics_avian_influenza_e.pdf) for more information on avian influenza-affected areas.

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communicated by:
ProMED-mail <promed@promedmail.org>

[This report describes a novel case of avian influenza (H10N8) virus infection, contracted in Jiangxi province of mainland China, by an immunocompromised elderly lady. Similarly to other avian influenza virus infections encountered so far (with the exception of H5N1 virus) there has been no onward transmission of infection to other related or unrelated humans. Circumstantial evidence suggests that the infection was contracted by exposure to domestic fowl in a local "wet" market. The patient died.

Further information is awaited..- Mod.CP

A HealthMap/ProMED-mail map can be accessed at: http://healthmap.org/r/7AX6.]

See Also

Avian influenza, human (155): China (HK, ZJ) WHO update 20131211.2110154
Avian influenza, human (154): China (HK,ZG) H7N9 father & son cases: corr. 20131212.2109558
Avian influenza, human (154): China (HK,ZG) H7N9 father & son cases 20131210.2103474
Avian influenza, human (153): China (HK) H7N9 quarantine extended 20131208.2099399
Avian influenza, human (152): China, H7N9 update 20131207.2098219
Avian influenza, human (151): China (HK) H7N9, 2nd case 20131206.2097187
Avian influenza, human (150): China (HK) H7N9, quarantine 20131204.2091984
Avian influenza, human (149): China, H7N9, live poultry markets, control 20131203.2090295
Avian influenza, human (148): China (HK) H7N9, 1st case 20131203.2087599
Avian influenza, human (147): China (Hong Kong) H7N9, first case 20131202.2087472
Avian influenza, human (145): China (ZJ) H7N9, new case, correction 20131130.2083933
Avian influenza, human (142): H7N9 vaccine development 20131118.2062079
Avian influenza, human (141): China: southern, H5N1 seroprevalence 20131117.2060088
Avian influenza, human (138): China (ZJ) H7N9 new case 20131111.2049326
Avian influenza, human (135): China (GD & ZG) H7N9 new cases, WHO 20131106.2041316
Avian influenza, human (134): China (GD), H7N9, child 20131105.2040007
Avian influenza, human (132): China, H7N9, live poultry markets 20131102.2034820
Avian influenza, human (126) - China: (ZJ) H7N9 re-emerges 20131024.2019232
Avian influenza, human (122): China (ZJ) H7N9, alert 20131015.2002724
Avian influenza, human (120): H7N9 genesis & source 20131010.1994804
Avian influenza, human (118): China, H7N9 low transmission potential 20131005.1982621
Avian influenza, human (111): China, H7N9, re-emergence prospects 20130822.1895874
Avian influenza, human (109) China: H5N1 & H7N9 emergence 20130822.1892436
Avian influenza (33): China (JS, ZH) LPAI H7N9, live markets, RFI 20130410.1636843
.....cp/sh/lm

別紙様式第２

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 2013年11月18日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	人C1-インアクチベーター		研究報告の公表状況	ProMed http://www.promedmail.org/direct.php?id=2055317	公表国 台湾	
販売名（企業名）	①ベリナートP ②ベリナートP静注用 （CSLベーリング株式会社）					

研究報告の概要	台湾の鳥インフルエンザ（H6N1型）のヒトへの感染が、メディアおよびジャーナルで報道/掲載された。 20歳の女性がインフルエンザ様疾患、および息切れのため、2013年5月に病院で受診した。同女性の咽頭スワブ検体から非亜型インフルエンザAウイルスを単離し、台北疾病管理センター（CDC）に移送して、遺伝子解析が行われた。その結果、H6N1亜型と同定された。感染源は確定されなかった。このウイルスの血球凝集素タンパク質にアミノ酸置換が見られ、そのためヒトα2,6シアル酸受容体に対する親和性が高くなっている可能性がある。 野生型鳥インフルエンザA（H6N1）ウイルスへのヒト感染が報告されたのは初めてである。

報告企業の意見	今後の対応
原料血漿の原産国は欧州および米国である。 製造工程にインフルエンザウイルスに有効な不活化/除去工程を有することから、インフルエンザウイルス（H6N1 型）に対する安全対策は取られていると考える。	今後とも新しい感染症に関する情報収集に努める所存である。

使用上の注意記載状況・その他参考事項等

ABJ2013Z04

Published Date: 2013-11-14 13:19:00
Subject: PRO/AH/EDR> Avian influenza, human (139): Taiwan, H6N1 epidemiological analysis
Archive Number: 20131114.2055317

AVIAN INFLUENZA, HUMAN (138): TAIWAN, H6N1 EPIDEMIOLOGICAL ANALYSIS
****

A ProMED-mail post
http://www.promedmail.org
ProMED-mail is a program of the
International Society for Infectious Diseases
http://www.isid.org

In this posting:
[1] Press report
[2] Journal publication

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[1] Press report
Date: Wed 13 Nov 2013
Source: Time [edited]
http://healthland.time.com/2013/11/13/first-case-of-new-bird-flu-identified-in-human-patient/

The latest version is called H6N1, and represents the 1st time that this strain of [avian influenza] has jumped from birds to people [see ProMED-mail post Avian influenza, human (86): Taiwan, H6N1 20130621.1785829]. Flu researchers are especially wary of wild avian species like migrating geese to run-of-the-mill chickens at local poultry markets. These birds harbour a series of influenza strains that generally don't make the birds sick, but could cause serious disease in people if they jumped to human hosts. In recent years, more bird flu viruses that had never infected people before have been finding new human hosts.

Last spring [2013], for example, scientists in China reported the 1st human cases of H7N9 infections. These viruses previously circulated among birds, but mutations helped them to survive and sicken people as well.

Now, researchers in Taiwan say another avian flu strain, H6N1, may have made the jump as well. Reporting in the journal Lancet [see abstract in [2] below], scientists describe what they found when they analyzed a throat swab from a 20-year-old woman who came to the hospital with shortness of breath and flu symptoms. When they sequenced the virus in her sample, they found it was very similar to H6N1 strains that have been found in chickens on the island since the 1970s, with one exception: this H6N1 had a mutation that gave it the ability to stick to human cells and gain entry, causing infection. Specifically, the mutation helped the virus to bind to cells in the human upper airway -- a good place for viruses to attach after they are inhaled through the nasal passages.

The woman, a clerk in a deli who did not have direct contact with raw meats or poultry, recovered. Scientists at the Centers for Disease Control (CDC) in Taiwan identified and tested 36 of her close contacts, including her brother, mother, neighbors, work colleagues, and doctors who cared for her at the hospital. Only 6 had fever and respiratory symptoms similar to the woman's; none showed signs of persistent infection with the virus.

While it's too early to tell how virulent H6N1 may be in people, the fact that this bird flu mutated and gained the ability to infect people is concerning, say public health experts. The development suggests that some of the H6N1 strains circulating among poultry in Taiwan now have the ability to make people sick. That's an unstable situation, since additional mutations could make the virus either more dangerous to people or make it more innocuous; it's all a matter of chance. "As these viruses continue to evolve and accumulate changes, they increase the potential risk of human infection," said Dr Ho-Sheng Wu, from the CDC in Taiwan and one of the study's co-authors. Coupled with the shift of H7N9 from birds to people last flu season [2012-2013], this latest case suggests that it makes sense to increase surveillance of the influenza strains circulating among birds for clues about the next potential

viral flu threat.

[Byline: Alice Park]

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[2] Journal publication
Date: Thu 14 Nov 2013
Source: The Lancet [edited]
http://www.thelancet.com/journals/lanres/article/PIIS2213-2600(13)70221-2/fulltext

Ref: Sung-Hsi Wei, Ji-Rong Yang, Ho-Sheng Wu, et al: Human infection with avian influenza A H6N1 virus: an epidemiological analysis. The Lancet Respiratory Medicine, early online publication, 14 Nov 2013; doi:10.1016/S2213-2600(13)70221-2

Summary

Background: Avian influenza A H6N1 virus is one of the most common viruses isolated from wild and domestic avian species, but human infection with this virus has not been previously reported. We report the clinical presentation, contact, and environmental investigations of a patient infected with this virus, and assess the origin and genetic characteristics of the isolated virus.

Methods: A 20-year-old woman with an influenza-like illness presented to a hospital with shortness of breath in May 2013. An unsubtyped influenza A virus was isolated from her throat-swab specimen and was transferred to the Taiwan Centres for Disease Control (CDC) for identification. The medical records were reviewed to assess the clinical presentation. We did a contact and environmental investigation and collected clinical specimens from the case and symptomatic contacts to test for influenza virus. The genomic sequences of the isolated virus were determined and characterised.

Findings: The unsubtyped influenza A virus was identified as the H6N1 subtype, based on sequences of the genes encoding haemagglutinin and neuraminidase. The source of infection was not established. Sequence analyses showed that this human isolate was highly homologous to chicken H6N1 viruses in Taiwan and had been generated through interclade reassortment. Notably, the virus had a G228S substitution in the haemagglutinin protein that might increase its affinity for the human alpha 2-6 linked sialic acid receptor.

Interpretation: This is the 1st report of human infection with a wild avian influenza A H6N1 virus. A unique clade of H6N1 viruses with a G228S substitution of haemagglutinin have circulated persistently in poultry in Taiwan. These viruses continue to evolve and accumulate changes, increasing the potential risk of human-to-human transmission. Our report highlights the continuous need for preparedness for a pandemic of unpredictable and complex avian influenza.

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[As far as this moderator is aware there have been no further reports of human infection with avian influenza (H6N1) virus in Taiwan or elsewhere. It is unclear how the patient contracted the infection and there has been no report of onward human-to-human transmission of the virus. It is clear that modification of the haemagglutinin protein binding site alone is not sufficient to endow this avian influenza virus with ability to spread in the human population. In the absence of other information it can only be concluded that this particular individual by habit or genetic constitution was particularly susceptible to avian influenza virus infection at a particular time in her life. - Mod.CP]

See Also

Avian influenza, human (138): China (ZJ) H7N9 new case 20131111.2049326
Avian influenza, human (137): Cambodia (KP) H5N1 20131109.2046967
Avian influenza, human (136): Cambodia (PO) H5N1, WHO 20131106.2041724
Avian influenza, human (131): Cambodia (BA) H5N1 20131101.2034016
Avian influenza, human (124): Cambodia (KT) H5N1, WHO 20131023.2016517
Avian influenza, human (116): Cambodia, new cases 20130919.1956667

Avian influenza, human (113): Cambodia (PP) 20130904.1923386
Avian influenza, human (110): Cambodia (KN) H5N1, new case 20130822.1895496
Avian influenza, human (107): Cambodia, H5N1, update 20130813.1879077
Avian influenza, human (95): Cambodia (PY) H5N1 20130712.1822212
Avian influenza, human (92): Cambodia (KP) H5N1, fatal 20130702.1803875
Avian influenza, human (86): Taiwan, H6N1 20130621.1785829
Avian influenza, human (58): (Taiwan ex China) H7N9 update 20130424.1669273
Avian influenza, human (23): Cambodia (KP) update 20130404.1621699
Avian influenza, human (12): Cambodia: (KM) fatal 20130227.1562508
Avian influenza, human (10): Cambodia 20130221.1553693
Avian influenza, human (09): Cambodia, Egypt, China, WHO 20130219.1549714
Avian influenza, human (08): Cambodia (KC) susp. 20130218.1548448
Avian influenza, human (06): Cambodia (KT) 20130213.1541230
Avian influenza, human (04): Cambodia, 6th case, fatal 20130208.1535664
Avian influenza, human (03): Cambodia, WHO update 20130202.1526316
Avian influenza, human (02): Cambodia, children, fatal, MOH/WHO 20130129.1519720
Avian influenza (07): Cambodia (TA) poultry, OIE 20130128.1518643
Avian influenza, human (01): Cambodia, fatalities 20130125.1514597
.....mpp/lm/cp/mj/lm

医薬品　研究報告　調査報告書

<table>
<tr><td colspan="2">識別番号・報告回数</td><td></td><td>報告日</td><td>第一報入手日
2013年10月28日</td><td colspan="2">新医薬品等の区分
該当なし。</td><td rowspan="3">総合機構処理欄</td></tr>
<tr><td colspan="2">一　般　的　名　称</td><td>別紙のとおり。</td><td rowspan="2">研究報告の
公表状況</td><td rowspan="2">Science Insider, 2013; October 21</td><td rowspan="2">公表国
マレーシア</td></tr>
<tr><td colspan="2">販売名（企業名）</td><td>別紙のとおり。</td></tr>
<tr><td rowspan="2">研究報告の概要</td><td colspan="5">問題点：マレーシアで、デング熱の原因ウイルスであるデングウイルスに関し、新たな血清型を有するウイルスが検出された。

デング熱の原因ウイルスであるデングウイルスに関し、2007年にマレーシアで発生したアウトブレイクの際に採取された検体の中から、4種の既知の血清型のウイルスとは異なる型のデングウイルスが検出された。同ウイルスの遺伝子配列を確認した結果、これまでに知られる血清型のウイルスとは系統発生学的に異なることが判明し、実験的にサルから得られた同ウイルスに対する抗体もこれまでに知られる血清型のウイルスとは明らかに異なるものであった。
今のところ、新たな血清型のデングウイルスは、上記の1回のアウトブレイクにおいてのみ検出されている</td><td rowspan="2">使用上の注意記載状況・
その他参考事項等

記載なし。</td></tr>
<tr><td colspan="3">報告企業の意見</td><td colspan="3">今後の対応</td></tr>
<tr><td colspan="3">別紙のとおり。</td><td colspan="3">今後とも関連情報の収集に努め、本剤の安全性の確保を図っていきたい。</td><td></td></tr>
</table>

MedDRA/J ver. 16.1

別紙

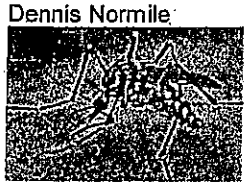
一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免役グロブリン、⑤人免役グロブリン、⑥乾燥ペプシン処理人免疫グロブリン、⑦乾燥スルホ化人免疫グロブリン、⑧乾燥スルホ化人免疫グロブリン、⑨乾燥スルホ化人免疫グロブリン、⑩乾燥スルホ化人免疫グロブリン、⑪乾燥スルホ化人免疫グロブリン*、⑫乾燥濃縮人活性化プロテインC、⑬乾燥濃縮人血液凝固第Ⅷ因子、⑭乾燥濃縮人血液凝固第Ⅷ因子、⑮乾燥濃縮人血液凝固第Ⅷ因子、⑯乾燥濃縮人血液凝固第Ⅸ因子、⑰乾燥濃縮人血液凝固第Ⅸ因子、⑱乾燥濃縮人血液凝固第Ⅸ因子、⑲乾燥抗破傷風人免疫グロブリン、⑳抗 HBs 人免疫グロブリン、㉑抗 HBs 人免疫グロブリン、㉒抗 HBs 人免疫グロブリン、㉓トロンビン、㉔フィブリノゲン加第ⅩⅢ因子*、㉕フィブリノゲン加第ⅩⅢ因子、㉖乾燥濃縮人アンチトロンビンⅢ、㉗ヒスタミン加人免疫グロブリン製剤、㉘人血清アルブミン*、㉙人血清アルブミン*、㉚乾燥ペプシン処理人免役グロブリン*、㉛乾燥濃縮人アンチトロンビンⅢ
販売名（企業名）	①献血アルブミン 20 “化血研”、②献血アルブミン 25 “化血研”、③人血清アルブミン “化血研” *、④ガンマーグロブリン筋注 450mg/3mL「化血研」、⑤ガンマーグロブリン筋注 1500mg/10mL「化血研」、⑥献血グロブリン注射用 2500mg「化血研」、⑦献血ベニロンーⅠ静注用 500mg、⑧献血ベニロンーⅠ静注用 1000mg、⑨献血ベニロンーⅠ静注用 2500mg、⑩献血ベニロンーⅠ静注用 5000mg、⑪ベニロン*、⑫注射用アナクトC2,500 単位、⑬コンファクトF注射用 250、⑭コンファクトF注射用 500、⑮コンファクトF注射用 1000、⑯ノバクトM静注用 400 単位、⑰ノバクトM静注用 800 単位、⑱ノバクトM静注用 1600 単位、⑲テタノセーラ筋注用 250 単位、⑳ヘパトセーラ筋注 200 単位/mL、㉑ヘパトセーラ筋注 200 単位/1mL、㉒ヘパトセーラ筋注 1000 単位/5mL、㉓トロンビン “化血研”、㉔ボルヒール*、㉕ボルヒール組織接着用、㉖アンスロビンP500 注射用、㉗ヒスタグロビン皮下注用、㉘アルブミン 20%化血研*、㉙アルブミン 5%化血研*、㉚静注グロブリン*、㉛アンスロビンP1500 注射用
報告企業の意見	デング熱はデングウイルス感染を原因とする感染症である。デングウイルスは 40～60nm の球形、核酸は一本鎖 RNA、エンベロープを有するウイルスで、蚊によって媒介され、これまでに 1～4 型の 4 つの血清型が知られている。 今回の報告は、マレーシアで、これまでに知られている 4 つ血清型とは異なる新たな血清型のデングウイルスが検出された、との報告である。 上記製剤の製造工程には、冷エタノール分画工程、ウイルス除去膜ろ過工程及びペプシン処理工程といった原理の異なるウイルスクリアランス工程が導入されており、各工程のウイルスクリアランス効果は「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（医薬発第 1047 号、平成 11 年 8 月 30 日）」に基づく、モデルウイルスを用いたウイルスプロセスバリデーションにより確認されている。今回報告した新規デングウイルスのモデルウイルスには、エンベロープの有無、核酸の種類等から、ウシウイルス性下痢ウイルス（BVDV）が該当すると考えられるが、上記工程の BVDV クリアランス効果については上記バリデーションにより確認されている。また、これまでに上記製剤による新規デングウイルスへの感染報告例は無い。 以上の点から、上記製剤はデング熱に対する安全性を確保していると考える。

*：現在製造を行っていない

INF2013-008

Published on *Science/AAAS | News* (http://news.sciencemag.org)

Article Title:
First New Dengue Virus Type in 50 Years
October 21, 2013
Dennis Normile



Centers for Disease Prevention and Control

Spreader. An *Aedes aegypti* mosquito, a major carrier of the Dengue fever virus.

BANGKOK—Scientists have discovered a new type of the virus that causes a centuries-old pestilence, dengue. The surprising find, announced at a major dengue conference here today, is bound to complicate efforts to develop a vaccine against a tropical disease that is becoming a more pervasive global menace. But it could shed light on where the pathogen came from and whether it is evolving into a greater threat. The finding "may change the way we think about dengue virus evolution and emergence," says Duane Gubler, a dengue expert at the Duke-NUS Graduate Medical School in Singapore.

There is no vaccine or drug against dengue, which is spread by mosquitoes and causes fever and sometimes excruciating joint pain and muscle aches. Patients typically recover on their own, though severe cases need medical support. Occasionally, the illness progresses to dengue hemorrhagic fever, a potentially fatal complication in which blood leaks through vessel walls. A dengue infection confers lifetime immunity to that particular type. But subsequent infection with a second type increases the likelihood of serious illness. With that in mind, vaccine developers have strived to protect against all four types simultaneously.

That may have gotten more challenging. By chance, researchers screening dengue viral samples found a virus collected during an outbreak in Malaysia's Sarawak state in 2007 that they suspected was different from the four original serotypes. They sequenced the virus and found that it is phylogenetically distinct from the other four types. Experiments found that monkey antibodies produced against the new type differ significantly from those resulting from the previously known dengue viruses. "We discovered and characterized a new dengue serotype," announced Nikos Vasilakis, a virologist at University of Texas Medical Branch in Galveston, here today at the Third International Conference on Dengue and Dengue Haemorrhagic Fever [1].

"They've done a very good job in characterizing the virus, and it's convincing that it is distinct from the other four," says Thomas Scott, a dengue expert at the University of California, Davis.

What it may mean for controlling dengue is unclear. So far, dengue 5 has been linked to only one outbreak in humans. Vasilakis suspects that it is circulating, possibly among macaques, in the forests of Sarawak. If it spreads, it could make human vaccine development more challenging. "We don't need another complication in controlling dengue," Scott says.

Current efforts to rein in the disease are falling short. In talks here today, researchers from Malaysia, the Philippines, and Thailand said that despite control programs launched in the 2000s, dengue cases are increasing, though the death toll is down thanks to better management of severe cases. "Dengue is spreading from urban to rural areas and to countries, such as Nepal, where it has not been seen before," said Samlee Plianbangchang, the World Health Organization Southeast Asia regional director. The annual global incidence, close to 390 million cases, is about three times [2] the burden previously estimated, researchers reported in April in *Nature*.

Despite a recent setback [3] in vaccine development, Plianbangchang said that projects in the pipeline have researchers "looking forward to a vaccine in the near future." But he adds that a vaccine will complement, not replace, efforts to contain dengue through mosquito control and public awareness. "Dengue will be with us for many years and could get worse," he said.

Links:
[1] http://www.dengue2013bangkok.com/home/index/en
[2] http://news.sciencemag.org/biology/2013/04/dengue-cases-three-times-higher-thought
[3] http://news.sciencemag.org/health/2012/09/mixed-results-dengue-vaccine-trial

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
				2013. 9. 6	該当なし	

一般的名称	解凍人赤血球液	研究報告の公表状況	Emerging Infectious Disease Journal, Vol.19 No.9; Available from: http://wwwnc.cdc.gov/eid/article/19/9/12-1884_article.htm	公表国 インド
販売名(企業名)	解凍赤血球濃厚液「日赤」(日本赤十字社) 照射解凍赤血球濃厚液「日赤」(日本赤十字社) 解凍赤血球-LR「日赤」(日本赤十字社) 照射解凍赤血球-LR「日赤」(日本赤十字社) 解凍赤血球液-LR「日赤」(日本赤十字社) 照射解凍赤血球液-LR「日赤」(日本赤十字社)			

研究報告の概要

○インド、バンディプル・トラ保護区におけるキャサヌル森林病(KFD)の拡大、2012－2013年
1956年にインドで発見されたKFDウイルス(フラビウイルス科フラビウイルス属)は、自然界でサルとダニの間で感染環が維持されている。ヒトが感染した場合、高熱、前頭部痛、激しい筋肉痛を呈し、鼻腔、咽頭、歯肉、時に消化管からの出血を来たす。KFDはインドのKarnataka州Malnad地域の5地区(Shimoga、Chikkamagalore、Uttara Kannada、Dakshina Kannada及びUdupi)に特有であり、毎年1～5月にかけて100～500人が罹患する。
2012年11月、Karnataka州Chamarajanagara地区のバンディプル国立公園で、サル12匹の死亡が報告され、それと同時にトラ保護区において病気のサルを処分した6人が典型的なKFD症状を呈し入院した。病原体特定のために、サルの剖検試料、感染疑い患者の血清サンプル及びダニを採取し、国立ウイルス研究所にてPCRを用いてKFDVの検査を行った。また、Chamarajanagara地区に隣接する地域(Tamil Nadu州及びKerala州)におけるサル、感染疑い患者、ダニのサンプルについても検査した。その結果、ヒトの21サンプル中12サンプル、サルの9サンプル中4サンプル及びダニの14プール中2プールが陽性(1プールは弱陽性)であった。サンプルから得られたKFDV塩基配列は、標準株P9605に95.8－98.1%の一致を示した。
この調査結果は、KFDが既知の流行地以外の地域で発生していることを裏付ける。Chamarajanagara地区、Tamil Nadu州及びKerala州におけるKFDVの検出は、インドの多くの常緑・半常緑森林地帯にウイルスが存在することを意味する。これまで組織化されたサーベイランスが行われてこなかったため、見過ごされた症例が存在する可能性がある。

報告企業の意見	今後の対応
インドにおいて、キャサヌル森林病の既知の流行地以外の地域(Chamarajanagara地区、Tamil Nadu州及びKeraka州)で採取されたサル、ヒト及びダニのサンプルからキャサヌル森林病ウイルスが検出されたとの報告である。	日本赤十字社では、輸血感染症対策として問診時に海外滞在歴の有無を確認し、帰国(入国)後4週間は献血不適としている。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。

使用上の注意記載状況・その他参考事項等

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

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

lacked adjustment for confounding, we think that the results of Bacci et al. (*1*) should be interpreted with caution. Furthermore, a large clinical study from 2008 concluded that *C. difficile* type 078, which is the most frequently found binary toxin positive non-027 strain, was not associated with a high all-cause mortality rate (*3*). A more recent publication confirmed this finding (*4*). Therefore, in our opinion, there is currently no convincing epidemiologic proof that binary toxin is a marker for infection with virulent *C. difficile*.

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Spread of Kyasanur Forest Disease, Bandipur Tiger Reserve, India, 2012–2013

To the Editor: Kyasanur Forest disease virus (KFDV; family *Flaviviridae*, genus *Flavivirus*) was first recognized in 1956 in Shimoga District, Karnataka State, India (*1*). The natural cycle of KFDV involves 2 monkey species–black-faced langurs (*Semnopithecus entellus*) and red-faced bonnet monkeys (*Macaca radiata*)–and various tick species (genus *Haemaphysalis*). Monkeys become infected with KFDV through the bite of infected ticks; the virus is then transmitted to other ticks feeding on infected monkeys. KFDV infection causes severe febrile illness in some monkeys. When infected monkeys die, ticks drop from the body, thereby generating hot spots of infectious ticks that further spread the virus. In the enzootic state, KFDV circulates through small mammals (e.g., rodents, shrews, ground birds) and ticks (*2*).

Humans can also be infected with KFDV. In humans, the disease causes high fever, frontal headache, and severe myalgia, followed by bleeding from the nasal cavity, throat, gingivae, and, in some cases, gastrointestinal tract (*3*). In the natural KFDV cycle, humans are dead-end hosts.

KFD is unique to 5 districts (Shimoga, Chikkamagalore, Uttara Kannada, Dakshina Kannada, and Udupi) in the Malnad region of Karnataka State, India, where each year during January–May, 100–500 persons are affected by the disease (*2*,*4*). During December 2011–March 2012, a total of 215 suspected KFD case-patients were identified in 80 villages in Shimoga District; laboratory testing confirmed that 61 (28%) were infected with KFDV (*5*).

In November 2012, the deaths of 12 monkeys in Bandipur National Park, Chamarajanagara District, Karnataka State, were reported. At the same time, 6 humans from Mole Hole village and Madhur colony in the Bandipur Tiger Reserve who handled and incinerated the sick monkeys were reported to have clinical signs and symptoms typical of KFD (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/9/12-1884-Techapp1.pdf). The monkey handlers (20–55 years of age) were admitted to the local hospital in Gundlupet Taluk. Monkey autopsy specimens, serum samples from suspected human case-patients, and tick pools were collected by staff from the Virus Diagnostic Laboratory in Shimoga. The samples were sent to the National Institute of Virology in Pune for determination of the etiologic agent. Additional samples from humans with suspected KFDV infection, monkeys, and tick pools were received from Chamarajanagar District and adjoining border areas of Tamil Nadu State and Kerala State (Table).

Monkey brain and liver and tick pools were sonicated in 600 mL of Minimum Essential Media (GIBCO/BRL, Life Technologies, Grand Island, NY, USA), and 400 mL of media was added to the homogenate. TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) was used to perform RNA extraction as described (*6*).

Samples were tested for KFDV by nested reverse transcription PCR (RT-PCR) and real-time RT-PCR as described (*6*); 12 of 21 human samples and 4 monkey samples were positive (Table). Two of 14 tick pools screened for KFDV by real-time RT-PCR were positive; however, 1 was weakly positive (Table). The PCR-amplified products were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and then sequenced. KFDV sequences from the samples showed 95.8%–98.1% similarity with prototype strain KFDV P9605. This finding supports the earlier conclusion that a high level of conservation exists for KFDV sequences (*7*). The phylogenetic tree formed 2 clades: the first

Table. Real-time reverse transcription PCR and nested reverse transcription PCR results for specimens screened for Kyasanur Forest disease virus, India, November 2012–May 2013*

Date of sample collection	Location of sample collection	No. samples positive/no. total		
		Human	Monkey	Tick pools
2012 Nov	Maddur Forest Range, Bandipur Tiger Reserve, Chamarajanagara District, Karnataka State	4/6	3/7	–
2013 Jan	Chamarajanagara District, Karnataka State	7/13	–	0/7
2013 Jan	Nilgiri, Tamil Nadu State	0/1	1/2	0/5
2013 Feb	Chamarajanagara District, Karnataka State	–	–	1/2
2013, May	Wayanad District, Kerala State	1/1	–	–
Total no. positive samples		12/21	4/9	1/14

*–, no samples from the area.

included mainly KFDV sequences from 1957–2006, the second included KFDV sequences (human and monkey) from Chamarajanagara District (online Technical Appendix Figure 2).

KFDV has not been detected previously in Chamarajanagara District, the location of Bandipur National Park. Affected areas in the district share a border with Mysore District (Karnataka State), Kerala State, and Tamil Nadu State. In addition,we subsequently found monkey samples from Nilgiri, Tamil Nadu, to be positive for KFDV.

The human case-patients from Chamarajanagara District were mainly forest workers involved in the incineration of the dead monkeys. Infection among these workers indicates that they did not follow appropriate biosafety procedures while handling the infected animals.

Our findings confirm that KFD has occurred outside the districts in Karnataka State where KFDV is known to be endemic. A hemagglutination inhibition antibody survey conducted during December 1988–January 1989 (*8*) indicated the possible existence of this disease in other regions of India. The presence of KFD becomes noticeable when enzootic infections occur and sentinel animals, like monkeys, start dying (*9*). Detection of KFDV in Chamarajanagara District, Tamil Nadu State (Nilgiri), and Kerala State indicates the presence of the virus in many evergreen and semi-evergreen forest areas of India. Infections in these areas may have been missed previously because of the lack of an organized surveillance system.

During the first week of December 2012, immediately after the KFD outbreak was confirmed, the Karnataka public health department vaccinated 322 persons, including villagers, forest officials, health workers, and members of local tribes in the Maddur Forest Range of Bandipur Tiger Reserve. Hot-spot areas caused by monkey deaths were dusted with malathion insecticide to kill ticks. In addition, to prevent additional human infections, epidemiologists recommended establishment of a health education campaign and the use of protective clothing and tick repellents, especially by persons frequently visiting forested areas.

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医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
				2013. 10. 10	該当なし	

一般的名称	人赤血球液	研究報告の公表状況		公表国
販売名(企業名)	赤血球濃厚液-LR「日赤」(日本赤十字社) 照射赤血球濃厚液-LR「日赤」(日本赤十字社)		Jia N, Zheng YC, Jiang JF, Ma L, Cao WC. N Engl J Med. 2013 Sep 19;369(12):1178-80. doi: 10.1056/NEJMc1303004.	中国

研究報告の概要

○ *Candidatus Rickettsia tarasevichiae*のヒト感染

2012年5月から8月まで、最近ダニに咬まれた合計251人の患者が中国北東部の病院を受診し、ダニ媒介感染症の検査を受けた。痂皮及び血清サンプルのPCR検査とシークエンシングにより、5人の患者が紅斑熱グループリケッチアの新種、*Candidatus Rickettsia tarasevichiae*に感染していることが明らかとなった。

系統発生解析により、この病原体は遺伝的に*R.canadensis*に近縁であることが示され、間接免疫蛍光分析では、IgMまたはIgG抗体が、紅斑熱グループリケッチアの2つの固有種*R.heilongjiangensis*及び*R.sibirica*に反応した。

5人の患者は12歳～56歳で、発熱、無力症、食欲不振、悪心、頭痛、痂皮及びリンパ節腫脹等を呈し入院した。検査の結果、白血球数の微増、AST上昇、タンパク尿等を示した。患者の1人は髄膜炎様症状を呈し、4日後に死亡した。いずれの患者も紅斑熱グループリケッチア感染の典型的徴候である発疹を伴わなかったため、当初誤診されていた。

患者の居住地の周囲でダニを採取し分析したところ、シュルツェマダニ453匹中46匹(10%)が*Candidatus R.tarasevichiae*陽性であった。

*Candidatus R.tarasevichiae*はロシアのシュルツェマダニから初めて検出された。今回の調査結果により、*Candidatus R.tarasevichiae*がヒトで感染症を引き起こす新興病原体であると特定された。また、リケッチア症は以前認識されていたよりも稀ではなく、発熱の誤診原因となりうる。

報告企業の意見	今後の対応
中国北東部で、ダニに咬まれて受診した251人中5人が、紅斑熱グループリケッチアの新種*Candidatus Rickettsia tarasevichiae*に感染していたとの報告である。	日本赤十字社では、発熱などの体調不良者を献血不適としているほか、リケッチア症の既往の申告がある場合は、完全に治癒して一定期間が経過するまで献血不適としている。今後も引き続き情報の収集に努める。

使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

325-bp *ompA* sequences revealed that the agent clustered with other *R. sibirica* subspecies and was most closely related to *R. sibirica* subspecies *sibirica* 246, which had been isolated from *Dermacentor nuttalli* in Russia (Fig. 1). In serum samples obtained from the patient, the titers of IgM and IgG antibodies against *R. sibirica* on indirect immunofluorescence assay increased from 1:32 and 1:64, respectively, in the acute phase to 1:4096 for IgM and for IgG in the convalescent phase.

The study of this case was approved by the institutional review board of the Chinese Academy of Military Medical Sciences. The patient provided written informed consent.

R. sibirica subspecies *sibirica* BJ-90 was initially isolated from *D. sinicus* in China in 1990,[2,3] and it was detected in *D. silvarum* in Russia.[4] Our case shows that this organism can cause human disease. Unlike patients infected with *R. sibirica* and *R. heilongjiangensis* in the same geographic region,[5] this patient was severely ill with multiorgan dysfunction. Further investigation of the epidemiologic and clinical features of *R. sibirica* subspecies *sibirica* BJ-90 is required to distinguish it from other known tickborne infections.

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Human Infection with *Candidatus Rickettsia tarasevichiae*

TO THE EDITOR: From May through August 2012, a total of 251 patients who had recent tick bites sought treatment at Mudanjiang Forestry Central Hospital in northeastern China and were tested for tickborne infections. Polymerase-chain-reaction testing followed by sequencing of eschar and blood samples showed that 5 patients were infected with *Candidatus Rickettsia tarasevichiae*, a new species of rickettsiae of the spotted fever group. Phylogenetic analysis based on either the citrate synthase gene or the outer-membrane protein A gene showed that the agent was genetically close to *R. canadensis* (see Fig. 1 in the Supplementary Appendix, available with the full text of this letter at NEJM.org), one of several "ancestral" rickettsiae that are suspected to be endosymbionts and nonpathogens.[1] In an indirect immunofluorescence assay, IgM or IgG antibodies reacted to two endemic species of rickettsiae of the spotted fever group, *R. heilongjiangensis* and *R. sibirica*.[2]

The study of these cases was approved by the institutional review board of the Chinese Academy of Military Medical Sciences. All patients provided written informed consent.

Characteristics of the five patients are shown in Table 1. All five patients had a recent tick bite and no documented immunocompromised conditions. Their ages ranged from 12 to 56 years (median, 30 years), and three were women. They were hospitalized with fever (in two patients), asthenia (in three patients), anorexia (in three

Table 1. Clinical Characteristics and Laboratory Test Results of the Five Patients with *Candidatus Rickettsia tarasevichiae* Infections on Admission.*

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age — yr	12	56	30	29	40
Sex	Female	Female	Female	Male	Male
Days between known tick bite and illness onset	9	10	2	13	17
Specimen source for PCR amplification	Eschar	Blood	Blood	Blood	Blood
Elevated temperature (°C)	No	No	No	Yes (39)	Yes (39)
Asthenia	No	Yes	Yes	No	Yes
Anorexia	No	Yes	Yes	No	Yes
Nausea	No	Yes	Yes	No	Yes
Headache	No	No	Yes	Yes	Yes
Rash	No	No	No	No	No
Eschar	Yes	Yes	Yes	No	No
Lymphadenopathy	Yes	No	Yes	No	No
Vomiting	No	No	Yes	No	Yes
Neck stiffness	No	No	Yes	No	Yes
Kernig's sign	No	No	No	No	Yes
Hospitalization — days	20	20	17	22	4
Hematologic test					
Leukocyte count — $\times 10^{-9}$/liter	10.5	5.4	9.8	11.6	11.8
Lymphocyte count — $\times 10^{-9}$/liter	4.0	2.7	2.2	1.4	1.6
Neutrophil count — $\times 10^{-9}$/liter	6.3	2.6	7.3	9.9	9.9
Hemoglobin — g/liter	143	143	122	163	143
Platelet count — $\times 10^{-9}$/liter	305	244	221	203	163
Biochemical test					
AST — U/liter	9.6	18.4	8.9	75.9	15.3
ALT — U/liter	15.5	34.3	14.4	44.4	19.9
Urinalysis					
Proteinuria	No	No	No	No	Yes
Cerebrospinal fluid measurements					
Leukocytes — per mm^3	NA	NA	0	NA	80
Lymphocytes — %	NA	NA	0	NA	60
Protein — g/liter	NA	NA	0.3	NA	0.6
Glucose — mmol/liter	NA	NA	3.8	NA	4.5

* ALT denotes alanine aminotransferase, AST aspartate aminotransferase, NA not available (not performed or not reported), and PCR polymerase chain reaction.

patients), nausea (in three patients), headache (in three patients), eschar (in three patients) (Fig. 2 in the Supplementary Appendix), and lymphadenopathy (in two patients). Patient 5 had meningitis-like manifestations such as vomiting, neck stiffness, and Kernig's sign. Coma, renal dysfunction, and respiratory acidosis then developed, and the patient died 4 days after admission to the hospital. Laboratory tests showed a slight increase in the leukocyte count (in three patients), an elevated level of aspartate aminotransferase (in one patient), proteinuria (in one patient), and an increase in the level of cerebrospinal fluid protein and leukocyte count in Patient 5 (Table 1). Since none of the patients presented with rash, which is considered to be a

typical sign of infections with species of rickettsiae of the spotted fever group in China,[3] their conditions were initially misdiagnosed, and they received penicillin G, leading to a prolonged hospitalization for approximately 20 days. To identify local natural foci, host-seeking ticks were collected around the patients' residences. A total of 46 of 453 *Ixodes persulcatus* ticks (10%) were positive for *Candidatus R. tarasevichiae*.

Candidatus R. tarasevichiae was initially detected in *I. persulcatus* ticks in various regions of Russia.[4] We identified the agent as an emerging pathogen causing human infection. These findings underscore the concept that rickettsioses are more common than previously realized and may be associated with misdiagnosed causes of fever globally.[5] Careful attention to clinical features and the use of molecular diagnostic tools could be helpful in establishing an etiologic diagnosis that may facilitate appropriate treatment and public health measures.

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CORRECTION

Global Health: Response to the AIDS Pandemic — A Global Health Model (June 6, 2013;368:2210-8). In Figure 1 (page 2212), the estimated HIV prevalence in Gabon should have been shown as 5.0 to <15.0%, rather than 15 to <28%. The article is correct at NEJM.org.

NOTICES

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19TH ANNUAL RODNEY APPELL UPDATE IN GYNECOLOGIC UROLOGY

The conference will be held in Georgetown, Grand Cayman, Feb. 13–15. Registration deadline is Jan. 31.

Contact Meeting Achievements, 232 E. 500 North, Valparaiso, IN 46383; or call (219) 465-1115; or e-mail polly@meetingachievements.com; or see http://urogynecologycourses.com.

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
				2013. 10. 10	該当なし	

一般的名称	人赤血球液	研究報告の公表状況		
販売名(企業名)	赤血球濃厚液-LR「日赤」(日本赤十字社) 照射赤血球濃厚液-LR「日赤」(日本赤十字社)		Kutsuna S, Kawabata H, Kasahara K, Takano A, Mikasa K. Am J Trop Med Hyg. 2013 Sep;89(3):460-1. doi: 10.4269/ajtmh.13-0187. Epub 2013 Jul 15.	公表国 日本

研究報告の概要

○日本における初の輸入回帰熱症例
ダニ媒介性回帰熱(TBRF)は世界のさまざまな地域で報告されているが、日本における回帰熱症例は報告されていなかった。2010年10月8日、生来健康な20歳女性が、周期性の発熱と下腿痛のために受診した。患者は9月1～8日までウズベキスタンに滞在し、その際、右大腿をダニに咬まれていた。帰国後10～12日間隔で4回の発熱があった。末梢血ギムザ染色によりボレリア属スピロヘータが示され、PCRを用いたflaB遺伝子のシークエンシング及び系統発生解析により、感染ボレリア種は*Borrelia persica*であると同定された。患者はTBRFと診断され、ミノサイクリン100mg×2/日を10日間投与後に、周期的な発熱は消失した。
この症例は日本における初のTBRF症例である。ソ連地域を含むTBRF風土病国から帰国した周期性の発熱を伴う患者において、TBRFを考慮しなくてはならない。

報告企業の意見	今後の対応
ウズベキスタンから帰国した日本人女性にダニ媒介性回帰熱が認められたとの報告である。	日本赤十字社では、輸血感染症対策として受付時に海外滞在歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、発熱などの体調不良者を献血不適としている。回帰熱の既往の申告がある場合は、完全に治癒して一定期間が経過するまで献血不適としている。今後も引き続き情報の収集に努める。

使用上の注意記載状況・その他参考事項等

赤血球濃厚液-LR「日赤」
照射赤血球濃厚液-LR「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

Am. J. Trop. Med. Hyg., 89(3), 2013, pp. 460–461
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JRC2013T-037

Case Report: The First Case of Imported Relapsing Fever in Japan

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Abstract. Tick-borne relapsing fever (TBRF) is endemic in discrete areas throughout the world; however, a domestic or imported case of relapsing fever has not been reported in Japan. Here, we report the first imported case. A previously healthy 20-year-old woman presented to our hospital on October 8, 2010, because of recurrent fever and lower leg pain. Before consultation, she had experienced four febrile episodes at 10–12-day intervals after returning from her stay in Uzbekistan from 1 to 8 September. Giemsa stain of peripheral blood showed *Borrelia* spirochetes. The spirochete was identified as *Borrelia persica* by sequencing of the amplicons of flaB using polymerase chain reaction and phylogenetic analysis. The patient was diagnosed with TBRF, and she completed a 10-day course of minocycline 100 mg twice daily. After treatment, her periodic fever subsided. Physicians should be aware of TBRF in patients with recurrent fever who have returned from TBRF-endemic countries, including areas of the former USSR.

Relapsing fever caused by spirochetes of the genus *Borrelia* is an acute febrile illness characterized by recurrent episodes of fever. These *Borrelia* spirochetes are transmitted to humans via the bite of an infected *Ornithodoros* tick (tick-borne relapsing fever; TBRF) or by contact with the body fluid of an infected human body louse (louse-borne relapsing fever). The TBRF is endemic in discrete areas throughout the world. Each *Borrelia* species that causes relapsing fever is different according to areas. The TBRF is caused by *Borrelia crocidurae* or *Borrelia duttonii* in Africa, whereas *Borrelia hermsii* or *Borrelia turicatae* in the North American continent. In central Asia, *Borrelia persica* or *Borrelia latyschewii* is the causative pathogen[1]; a domestic or imported case of relapsing fever has not been reported in Japan. Here, we report the first imported case.

A previously healthy 20-year-old woman presented to our hospital on October 8, 2010, because of recurrent fever and lower leg pain. Before consultation, she had experienced four febrile episodes at 10–12-day intervals. She had visited Rishton, a town in Fergana Province, Uzbekistan, from 1 to 8 September while working as a Japanese language volunteer. She stayed in a house with a thatched roof with a local family in Rishton and meals were mainly taken at home. She recalled being bitten by a tick on her right thigh. Four days after returning from Uzbekistan, she visited her primary care doctor because of high fever and lower leg pain. She was diagnosed with common cold, and was prescribed cefcapene pivoxil (CFPN-PI). After taking CFPN-PI, her fever promptly resolved, and she discontinued treatment with CFPN-PI. However, she experienced two further episodes of recurrent high fever and lower leg pain, at 12 and 22 days, respectively, after the first febrile episode. She took CFPN-PI during each episode, and her fever rapidly abated. During the fourth febrile episode, occurring 11 days after the third episode, she presented to our hospital for further evaluation.

On examination, her temperature was 39.8°C, her blood pressure was 112/70 mm of Hg, and her pulse rate was 90/min. Her physical examination revealed normal findings except for eschar on her right inner thigh. Laboratory tests revealed lymphopenia (1,110 μg/mL), elevated levels of C-reactive protein (195 mg/L) and alanine aminotransferase (69 IU/mL). Leukocytes were 7,290 μg/mL (73% of neutrophil and 15.2% of lymphocyte). Count of red blood cells and platelets were normal. Abdominal ultrasonography revealed mild splenomegaly. Blood culture was negative (BACTEC plus aerobic medium and anaerobic medium, Becton, Dickinson and Company, Franklin Lakes, NJ), and Giemsa stain of peripheral blood collected during the febrile phase showed helical bacteria suggestive of *Borrelia* spirochetes (Figure 1). Borrelial DNA was also detected in blood cultures and serum specimens obtained during the fourth febrile phase using *Borrelia*-specific flagellin gene (flaB)-based polymerase chain reaction (PCR).[2] The sequence was deposited in GenBank as accession no. AB781030. The spirochete was identified as *B. persica* by sequencing of the amplicons of flaB using PCR and phylogenetic analysis (sequence similarity was 99.6% to *B. persica* strain T [accession no. JF708953], whereas 86% to *Borrelia hispanica*, 84–85% to *B. duttonii* and *B. crocidurae*, 84% to *Borrelia recurrentis* strains). The post-treatment serum sample was negative in this PCR assay. The *Borrelia* was inoculated into culture bottles containing Barbour-Stoenner-Kelly medium with 10% rabbit serum (homemade); however, they did not propagate. The patient was diagnosed with relapsing fever, and she completed a 10-day course of minocycline (100 mg twice daily). Jarisch-Herxheimer syndrome did not occur. After treatment, her periodic fever subsided.

The TBRF is characterized by periodic fever with nonspecific symptoms such as headache, myalgia, arthralgia, and nausea. These symptoms last several days, followed by an interval without fever, followed by another episode of fever. If left untreated, patients usually experience 1 to 4 episodes of fever before the illness spontaneously resolves. The definitive diagnosis of TBRF is primarily based on the detection of *Borrelia* spirochetes in smears of peripheral blood collected during the febrile period. The PCR techniques (e.g., glpQ-based PCR) along with sequence analysis can often identify the infectious *Borrelia* species[3]; although the preferred treatment is doxycycline 100 mg twice daily for 10 days, we prescribed minocycline as an alternative treatment in this case. De Pierpont and others all reported that minocycline was as effective as doxycycline in areas with limited resources.[4]

Borrelia persica is frequently detected in Israel, Iran, and Jordan. However, the tick *Ornithodoros tholozani*, the

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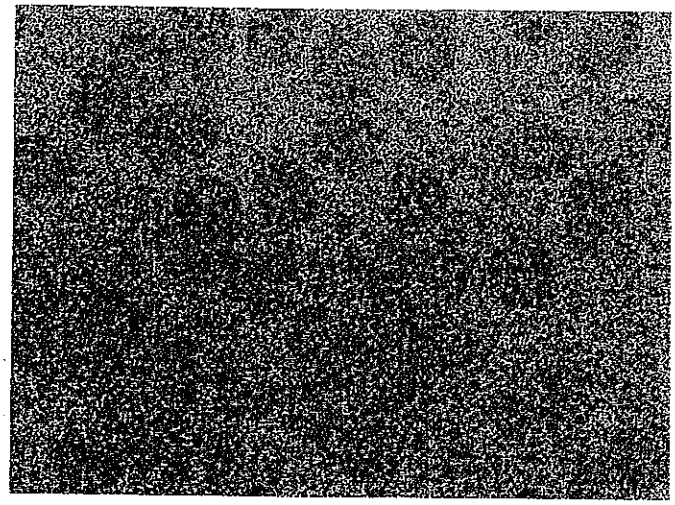

FIGURE 1. *Borrelia spirochaeta* in the Giemsa stain of the peripheral blood corrected at the febrile phase. This figure appears in color at www.ajtmh.org.

transmission vector of *B. persica*, is widely distributed throughout India, the southern countries of the former Union of Soviet Socialist Republics (USSR, including Kazakhstan, Kyrgizia, Tajikistan, Turkmenistan, and Uzbekistan), Middle East and Near East countries, Turkey, Egypt, and Cyprus.[1,5] To our knowledge, although the epidemiological evidence regarding TBRF is inadequate in the countries of the former USSR,[6] Colin de Verdiere and others[7] reported a case of TBRF caused by *B. persica* imported from Uzbekistan in 2011.

Several cases of imported TBRF have been reported throughout the world. Most are cases of disease imported to Europe from African countries[8–10]; our case is the first TBRF case in Japan imported from Uzbekistan. Tick-borne relapsing fever is usually benign and self-limiting, but there are severe complications such as meningoencephalitis caused by *B. crocidurae*.[11] Preferred treatment of TBRF in adults is tetracycline or doxycycline, and erythromycin, penicillin, or ceftriaxone can be used as alternatives. There may have been previously overlooked cases in Japan that resolved spontaneously or during treatment with prolonged courses of antibiotics.

Physicians should be aware of relapsing fever in patients with recurrent fever who have returned from TBRF-endemic countries, including areas of the former USSR. Diagnosis is primarily based on examination of peripheral blood smears. Where available, PCR is useful because of its sensitivity and ability to identify the causative species.

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感染症定期報告に関する今後の対応について

平成１６年度第５回
運営委員会確認事項
（平成１６年９月１７日）

１ 基本的な方針

運営委員会に報告する資料においては、

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

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

２ 具体的な方法

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

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

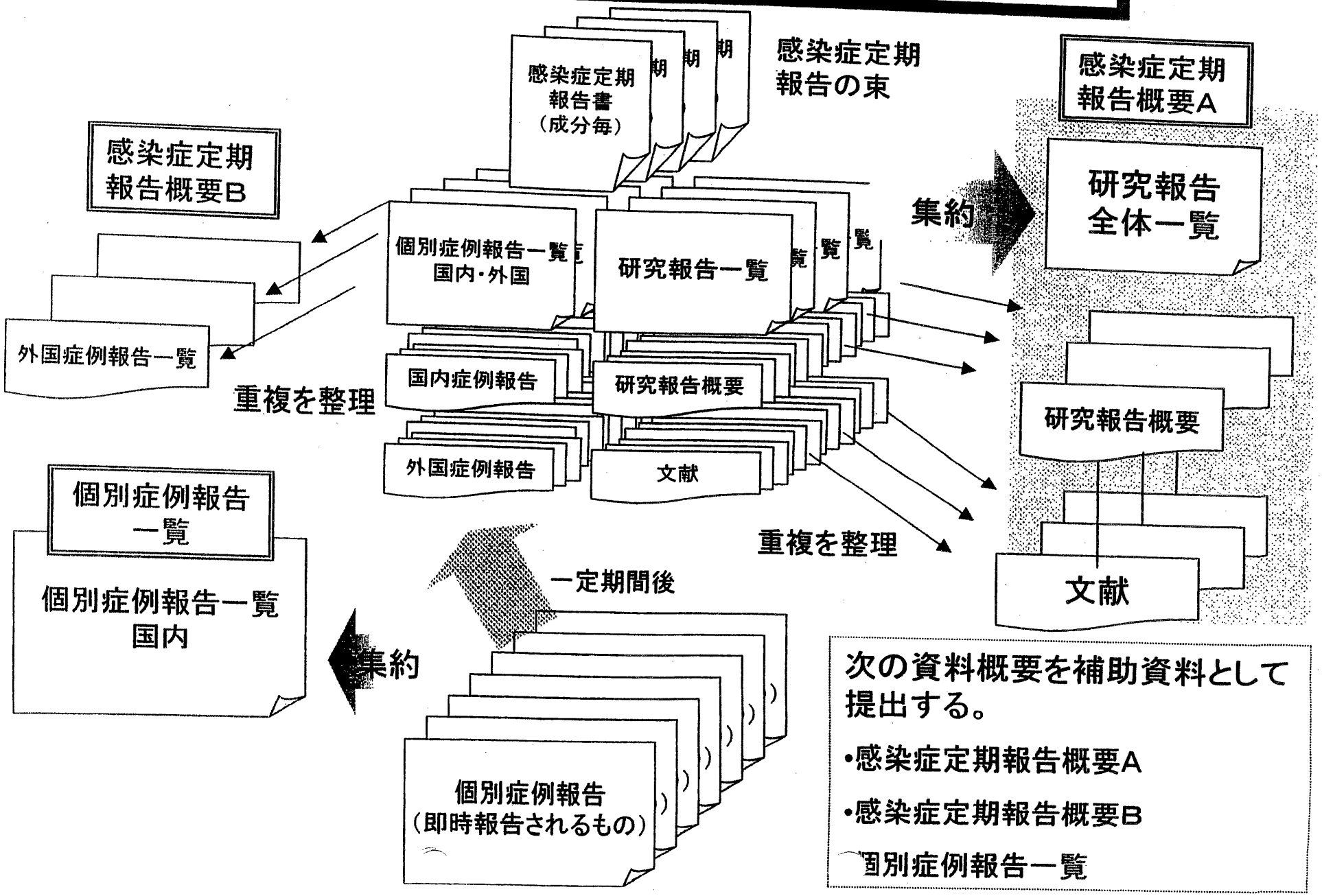
② 感染症発生症例報告のうち、発現国が「外国」の血漿分画製剤の使用による症例は、同一製品毎に報告期間を代表する感染症発生症例一覧（別紙様式第4）をまとめた**「資料概要B」**を事務局が作成し、送付する。

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

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

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

感染症定期報告・感染症個別症例報告の取り扱い



次の資料概要を補助資料として提出する。

- 感染症定期報告概要A
- 感染症定期報告概要B
- 個別症例報告一覧