平成 30 年度第１回運営委員会

資料１－２

Ａ　研究報告（詳細版）

平成３０年５月３０日

（平成３０年２月～平成３０年４月受理分）

別添3

感染症研究報告　調査報告書

調査報告書番号	KK-kaketsuken-20171122	文献ID	29152574
研究報告の題目	Human intestinal tract serves as an alternative infection route for Middle East respiratory syndrome coronavirus		

研究報告の概要

MERSCoVは、2012年に報告され、人で高い致死率(35%、2017/7現在)を示す呼吸器感染を起こす。しかし、感染伝播様式はあまり解明されていない。疫学的及びウイルス学的な知見により、著者らは人の消化器系からMERS-CoV感染が起こるという仮説をたて、検証した。患者の1/3は何らかの胃腸症状を呈する(嘔吐、下痢等)。
著者らは、人の初代腸上皮細胞、小腸移植片、腸オルガノイド(人工的に再構築された臓器)を用い、これらがMERS-CoVに高感受性であり、ウイルス複製が維持されることを証明した(呼吸器系よりも増殖が良好)。また、感染患者の糞便サンプルからMERS-CoVの腸管感染を同定した(複製過程のRNAを糞便サンプルから検出)。MERS-CoVは食事中をシュミレートした人工胃/小腸液に耐性を示した(絶食状態をシュミレートした人工胃液では酸性が強いので失活した)。極性化したCaco-2細胞(管腔側と基底側の特徴をもたせたCaco-2細胞培養で)、頂上(管腔側)側からのMERS-CoV感染は、基底側からの感染よりも効率的であった。さらに、MERS-CoVの胃内投与により、人DPP4(レセプターであり、呼吸器系だけでなく消化器系でも発現する)トランスジェニックマウスは致死的感染を起こした。組織学的検査により、投与されたマウス全てで腸管感染(小腸)が示された。腸管感染の進行により、ウイルスは肺組織に出現し、呼吸器感染を引き起こす。

・MERSの20%はラクダ(呼吸器系から多量のウイルスを排泄)との接触。ラクダ乳も感染源。乳中のウイルス生存性は高い。糞便中にもウイルス排泄。ラクダ肉も感染源とされる。
・人-人感染では飛沫感染は、患者から1～2mとされているが、韓国で発生した院内アウトブレークでは、直接接触は10%程度と推測された。よって、媒介物(器具、機材等)による感染が考えられた。媒介物による感染であるならば、腸管感染がもっとも可能性が高いと思われる。

報告企業の意見

中東呼呼吸器症候群(MERS)は、2012年に初めて報告された新種のコロナウイルスによる感染症で、 中東地域に居住または渡航歴のある者、あるいはMERS患者との接触歴のある者からの患者発生が継続的に報告されている。
MERSの原因病原体であるコロナウイルスは80～160nmの球形または楕円形で、核酸は一本鎖RNA、エンベロープを有する。
感染経路は、正確には分かっていないが、ヒトコブラクダが感染源の一つであると推定されている。その一方で、患者の中には動物との接触歴がない人も多く含まれており、家族間、医療機関における患者間、患者－医療従事者間など、濃厚接触者間での限定的なヒト―ヒト感染も一部報告されている。
今回、人の初代腸上皮細胞、小腸移植片、腸オルガノイドを用い、これらがMERS-CoVに対して感受性が高く、ウイルス複製が維持されることが証明された。さらに、MERS-CoVの胃内投与により、人DPP4トランスジェニックマウスは致死的感染を起こした。組織学的検査により、投与された全てのマウス全で腸管感染(小腸)が認められ、腸管感染の進行により、ウイルスは肺組織に出現し、呼吸器感染を引き起こすがこと示された。

今後の対応

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。

その他参考事項等

(注意)
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

VIROLOGY

Human intestinal tract serves as an alternative infection route for Middle East respiratory syndrome coronavirus

Jie Zhou,[1,2,3]* Cun Li,[2]* Guangyu Zhao,[4]* Hin Chu,[1,2,3] Dong Wang,[2] Helen Hoi-Ning Yan,[5] Vincent Kwok-Man Poon,[2] Lei Wen,[2] Bosco Ho-Yin Wong,[2] Xiaoyu Zhao,[2] Man Chun Chiu,[2] Dong Yang,[2] Yixin Wang,[2] Rex K. H. Au-Yeung,[5] Ivy Hau-Yee Chan,[6] Shihui Sun,[4] Jasper Fuk-Woo Chan,[1,2,3,7,8] Kelvin Kai-Wang To,[1,2,3,7,8] Ziad A. Memish,[9,10] Victor M. Corman,[11,12] Christian Drosten,[11,12] Ivan Fan-Ngai Hung,[13] Yusen Zhou,[4†] Suet Yi Leung,[5] Kwok-Yung Yuen[1,2,3,7,8†‡]

Middle East respiratory syndrome coronavirus (MERS-CoV) has caused human respiratory infections with a high case fatality rate since 2012. However, the mode of virus transmission is not well understood. The findings of epidemiological and virological studies prompted us to hypothesize that the human gastrointestinal tract could serve as an alternative route to acquire MERS-CoV infection. We demonstrated that human primary intestinal epithelial cells, small intestine explants, and intestinal organoids were highly susceptible to MERS-CoV and can sustain robust viral replication. We also identified the evidence of enteric MERS-CoV infection in the stool specimen of a clinical patient. MERS-CoV was considerably resistant to fed-state gastrointestinal fluids but less tolerant to highly acidic fasted-state gastric fluid. In polarized Caco-2 cells cultured in Transwell inserts, apical MERS-CoV inoculation was more effective in establishing infection than basolateral inoculation. Notably, direct intragastric inoculation of MERS-CoV caused a lethal infection in human DPP4 transgenic mice. Histological examination revealed MERS-CoV enteric infection in all inoculated mice, as shown by the presence of virus-positive cells, progressive inflammation, and epithelial degeneration in small intestines, which were exaggerated in the mice pretreated with the proton pump inhibitor pantoprazole. With the progression of the enteric infection, inflammation, virus-positive cells, and live viruses emerged in the lung tissues, indicating the development of sequential respiratory infection. Taken together, these data suggest that the human intestinal tract may serve as an alternative infection route for MERS-CoV.

INTRODUCTION

Middle East respiratory syndrome coronavirus (MERS-CoV) was identified as a novel zoonotic virus causing human respiratory infection in 2012 (*1*). As of July 2017, MERS-CoV has caused 2037 laboratory-confirmed infection cases, including 710 deaths, with a crude case fatality rate of about 35% (*2*). The clinical spectrum of MERS ranged from asymptomatic or mild respiratory disease to acute fulminant pneumonia with respiratory distress syndrome or multiorgan failure resulting in a fatal outcome. Common symptoms were fever, cough, and shortness of breath. Gastrointestinal symptoms were among the most commonly reported extrapulmonary clinical features of MERS; about one-third of MERS patients had gastrointestinal tract symptoms such as abdominal pain, nausea, vomiting, and diarrhea (*3*, *4*).

It has been reported that up to 20% of MERS cases are considered as primary infections due to contact with camels (*5*). MERS-CoV–infected camels, especially the juvenile ones, were found to shed a large amount of the virus from the upper respiratory tract (*6*). Camel milk may also play a role in the transmission of MERS-CoV. MERS-CoV could be detected in 41.7% of milk samples collected from lactating camels, which also actively shed the virus in nasal secretion and/or feces (*7*). In addition, MERS-CoV can survive in camel milk for a prolonged period (*8*). The consumption of unpasteurized camel milk was found to be a source of infection in some MERS patients (*9*). Thus, it is generally believed that primary infection, that is, MERS-CoV transmission from camel to human, is mediated via respiratory droplet and/or saliva during direct contact with camels or through consumption of camel milk or undercooked camel meat (*10*).

Meanwhile, a large proportion of MERS cases occurred in healthcare settings, in which respiratory droplets of MERS patients as well as direct or indirect contact have been postulated to be important for virus transmission (*3*, *11*). Theoretically, human-to-human virus transmission mediated by respiratory droplets (>5 μm in size) occurs within 1 to 2 m from a source patient. However, history of direct contact can be inferred in only about 10% of patients in the largest hospital-associated MERS outbreak reported so far, which occurred in the Republic of Korea, with 186 infection cases in 2015 (*12*). Most of the cases in this large-scale outbreak were those who shared the same healthcare environment without direct contact with MERS patients. Therefore, fomite transmission may explain a significant proportion of the infections (*12*). The virological evidence to support fomite transmission is that MERS-CoV remains stable at low temperatures and

[1]State Key Laboratory of Emerging Infectious Diseases, University of Hong Kong, Hong Kong, China. [2]Department of Microbiology, University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China. [3]Research Centre of Infection and Immunology, University of Hong Kong, Hong Kong, China. [4]State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China. [5]Department of Pathology, University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China. [6]Department of Surgery, Queen Mary Hospital, Hong Kong, China. [7]Carol Yu Centre for Infection, University of Hong Kong, Hong Kong, China. [8]Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, University of Hong Kong, Hong Kong, China. [9]Ministry of Health and College of Medicine, Alfaisal University, Riyadh, Kingdom of Saudi Arabia. [10]Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA. [11]Institute of Virology, Charité–Universitätsmedizin Berlin, Berlin, Germany. [12]German Centre for Infection Research (DZIF), Berlin, Germany. [13]Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong, China.
*These authors contributed equally to this work.
†These authors contributed equally to this work.
‡Corresponding author. Email: kyyuen@hku.hk

low humidity, and could be recovered after exposure to the environment for 48 hours (*13*). Accordingly, viral RNA can be extensively detected in the environmental surfaces in rooms of MERS patients (*14*). Given that fomite transmission may play a role in the outbreak in healthcare settings, acquisition of MERS-CoV infection via the gastrointestinal tract should be seriously considered. Collectively, in both camel-to-human transmission and human-to-human transmission, there is a possibility that humans may acquire MERS-CoV infection upon exposure to the virus through the gastrointestinal tract.

A protein intrinsic disorder–based model was previously established to classify transmission behaviors of coronaviruses by measuring the percentage of intrinsic disorder in the two major shell (matrix and nucleocapsid) proteins of coronaviruses (*15*). The analysis of protein sequences of these two proteins of MERS-CoV suggests that MERS-CoV belongs to a category of coronaviruses that have relatively hard inner and outer shells; therefore, it may persist in the environment for a prolonged period and may have an oral-fecal transmission ability (*16*). The bioinformatically predicted MERS-CoV survival upon environmental exposure has been experimentally corroborated (*13*). Here, we sought to elucidate whether the gastrointestinal tract could be an alternative infection route for MERS-CoV and whether the exposure of MERS-CoV to the gastrointestinal tract can subsequently lead to a respiratory infection. To this end, we performed in vitro studies in human primary intestinal epithelial cells, intestinal explants, polarized Caco-2 cells, and human intestinal organoids, as well as an in vivo study in human dipeptidyl peptidase 4 (hDPP4) transgenic mice.

RESULTS

Susceptibility of human primary intestinal epithelial cells and small intestine explants to MERS-CoV, and evidence of alimentary infection in clinical MERS patients

To address whether the human gastrointestinal tract could serve as an infection route for MERS-CoV, we examined the susceptibility of human primary intestinal epithelial cells to MERS-CoV. To this end, human primary intestinal epithelial cells were cultured for 1 week for differentiation. The differentiated epithelial cells were inoculated with MERS-CoV and were fixed at 24 hours post-infection (hpi) for immunofluorescence staining to identify the virus-positive cell. Almost all the inoculated intestinal epithelial cells highly expressed MERS-CoV NP, whereas no viral protein was detectable in mock-infected cells (Fig. 1A). In addition, the infected cells underwent significant membrane fusion and formed syncytia. Consistent with the intensive expression of viral protein, the viral load increased by more than 1 log in the MERS-CoV–inoculated epithelial cells (fig. S1). To further verify the infectivity of MERS-CoV in human intestinal epithelium, normal human small intestine from a surgical resection was obtained with informed consent and used for MERS-CoV inoculation. Despite tissue degradation, immunofluorescence staining explicitly revealed NP-positive enterocytes in the infected intestine (Fig. 1B) at 20 hpi. The infected enterocytes formed syncytia similar to those in the primary cells. Although only patchy areas of epithelium were infected, we observed an increased viral load of about 1 log in the Matrigel and medium, as well as in the infected explants (Fig. 1C). Thus, human primary intestinal epithelial cells and small intestine can be infected by MERS-CoV and support viral replication.

In an earlier study, we reported that 12 of 82 stool specimens of MERS patients were positive for MERS-CoV RNA (*17*), suggesting the possible MERS-CoV infection in the gastrointestinal tract of these patients. To gather more evidence of alimentary infection in MERS patients, these positive specimens were analyzed with a quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay for the detection of subgenomic RNA (sgRNA) of the nucleocapsid (N) gene, an intermediate in the replication cycle of MERS-CoV. Notably, N sgRNA was detected in the specimen and was confirmed by sequencing the qPCR product, which showed a typical sgRNA sequence with fused leader sequence, the predicted transcription regulatory site (TRS), and the body element of N gene (Fig. 1D). The presence of N sgRNA in the stool specimen suggested that MERS-CoV probably infected and replicated in the alimentary tract of the MERS patient. Together, the results in human primary intestinal epithelial cells and small intestine explants, together with the examination of the MERS patient's specimen, indicated that MERS-CoV can infect and replicate in human intestinal epithelium.

Highly productive MERS-CoV infection in human intestinal organoids

Generation of intestinal organoids from $LGR5^+$ tissue-resident adult stem cells in human intestines has been a major breakthrough in the past years. The differentiated intestinal organoids harbor all types of epithelial cells in human intestine and can simulate most morphological and functional properties of in vivo tissues (*18*). Therefore, three-dimensional cultured intestinal organoids, also known as intestinoids or mini-gut, have been used to model human diseases (*19*), including the infection of enteric viruses and bacteria (*20*, *21*).

We characterized MERS-CoV infectivity and replication kinetics in human intestinoids. At 48 hours after inoculation, viral loads increased by about 3 log units in the intestinoids and in the Matrigel and medium (Fig. 2A) after inoculation with a multiplicity of infection (MOI) of 1. The infected intestinoids developed progressive cytopathic effects over time. Thus, normalized viral loads in the infected intestinoids exhibited a constant increment within the 48-hour observation window (Fig. 2B). Accordingly, plaque assays revealed a significantly increased viral titer of 3 log units in the Matrigel and medium at 48 hpi (Fig. 2C). The infection efficiency of MERS-CoV in intestinoids, that is, the percentage of MERS-CoV–infected intestinal epithelial cells, was also examined with flow cytometry. At 2 hpi, about 5% of the cells within the intestinoids were MERS-CoV NP–positive. The percentage of NP-positive cells significantly increased to approximately 25% at 24 hpi (Fig. 2, D and E), suggesting a productive virus infection and spread in the intestinoids. The productive MERS-CoV infection in intestinoids was also evidenced by a strong signal of viral NP in the virus-inoculated intestinoids, albeit absent in the mock-infected organoids (Fig. 2F). In addition, we verified the expression of the MERS-CoV receptor DPP4, a major determinant for tissue tropism of MERS-CoV, in intestinal epithelial cells by immunofluorescence staining (fig. S2). Together, human intestinal organoids were highly susceptible to MERS-CoV and supported robust viral replication.

Viability of MERS-CoV in gastrointestinal fluids

The ability to retain viability in gastrointestinal fluids is a prerequisite for a microbe to establish infection in the human alimentary tract. In this regard, we assessed whether MERS-CoV can maintain infectivity in simulated human gastrointestinal fluids. As shown in Fig. 3, MERS-CoV rapidly lost most of the infectivity in fasted-state simulated gastric fluid (FaSSGF; pH 2.0). We assume that, in a real-life scenario, the virus is more likely to be exposed to fed-state gastrointestinal fluids because it is supposed to access the stomach during food intake. Thus,

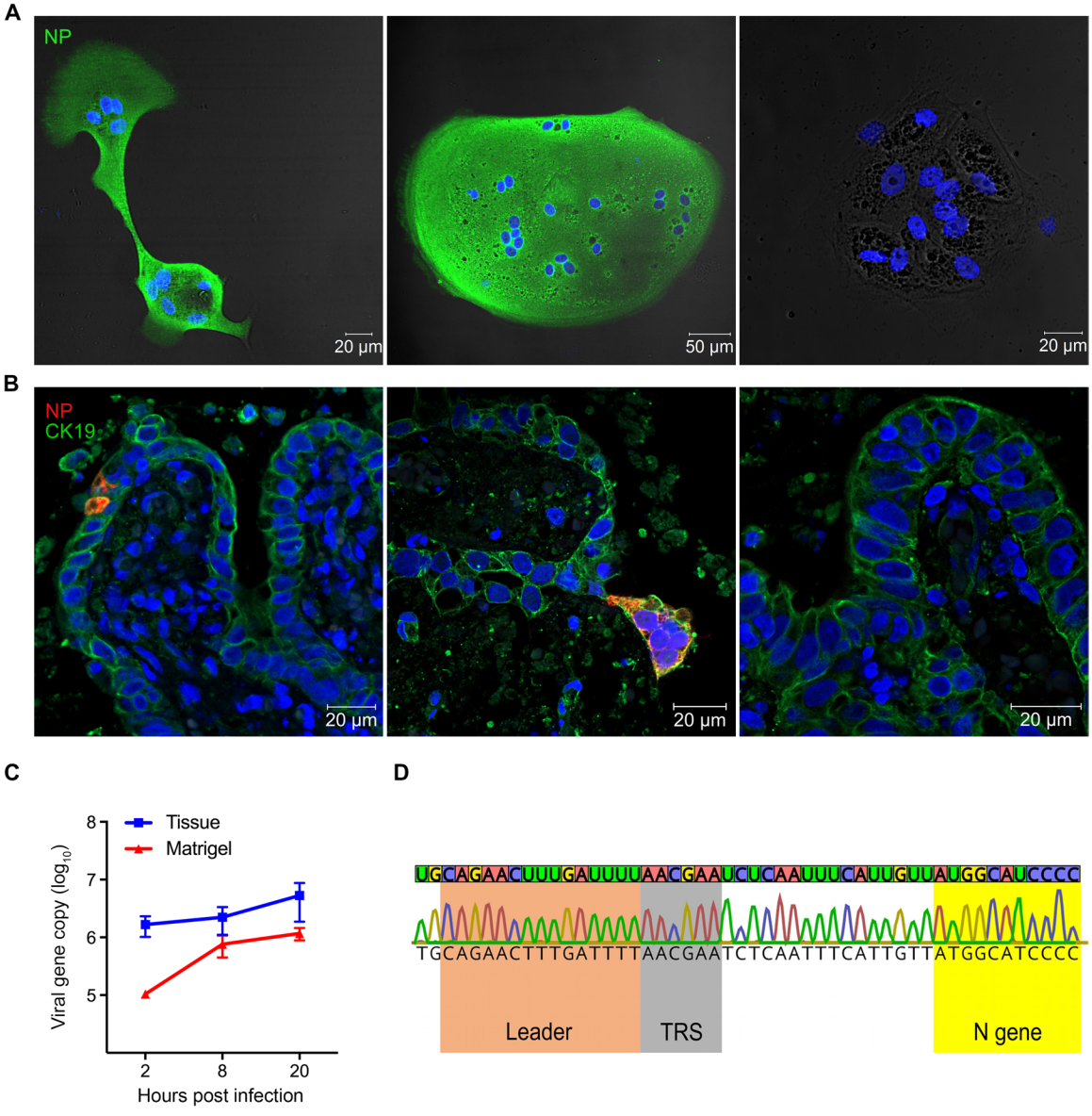


Fig. 1. Susceptibility of human primary intestinal cells, intestine explants to MERS-CoV, and identification of the replication intermediate in the stool specimen of a MERS patient. (**A**) Human primary intestinal cells were inoculated with MERS-CoV (left and middle) or mock-infected (right). At 24 hpi, cells were fixed and applied to immunofluorescence staining of MERS-CoV nucleocapsid protein (NP). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). The images show the representative results of one experiment. (**B** and **C**) Normal human small intestine explants were inoculated with MERS-CoV, as described in Materials and Methods. The data show representative results of the experiments independently performed twice. (B) The infected (left and middle) and mock-infected (right) explants were fixed at 20 hpi for immunofluorescence staining of MERS-CoV NP and the enterocyte marker CK19. Syncytia formation can be observed in the infected intestinal epithelium (middle). (C) At the indicated hpi, the explants (Tissue) and Matrigel together with culture medium (Matrigel) were harvested for the quantification of viral load. Data are means ± SD of viral loads in duplicated samples. (**D**) Chromatograph of the N gene sgRNA recovered from the stool specimen of a MERS patient show fusion of leader sequence, TRS, and N gene element.

we tested the infectivity of MERS-CoV after exposure to fed-state simulated gastric fluid (FeSSGF) or fed-state simulated intestinal fluid (FeSSIF), which contains a high concentration of bile salts that solubilize the lipid membrane of enveloped viruses. The results showed that MERS-CoV survived FeSSGF while being less tolerant to FeSSIF. Nevertheless, a small proportion of the virus can survive FeSSIF after treatment for 2 hours. As a control, EV71, a prototype human enterovirus, was generally resistant to all the tested gastrointestinal fluids. Another human coronavirus, hCoV-229E, which often causes mild respiratory infection, showed a comparable sensitivity to FaSSGF and FeSSGF to MERS-CoV, but was much less resistant to the intestinal fluid than MERS-CoV. All viruses exhibited considerable stability in Dulbecco's modified Eagle's medium (DMEM) over a period of 2 hours. Collectively, MERS-CoV was able to resist, to some extent, the digestive enzymes and bile salts in the human gastrointestinal tract, although the virus was less tolerant to the high acidity of fasted-state gastric fluid.

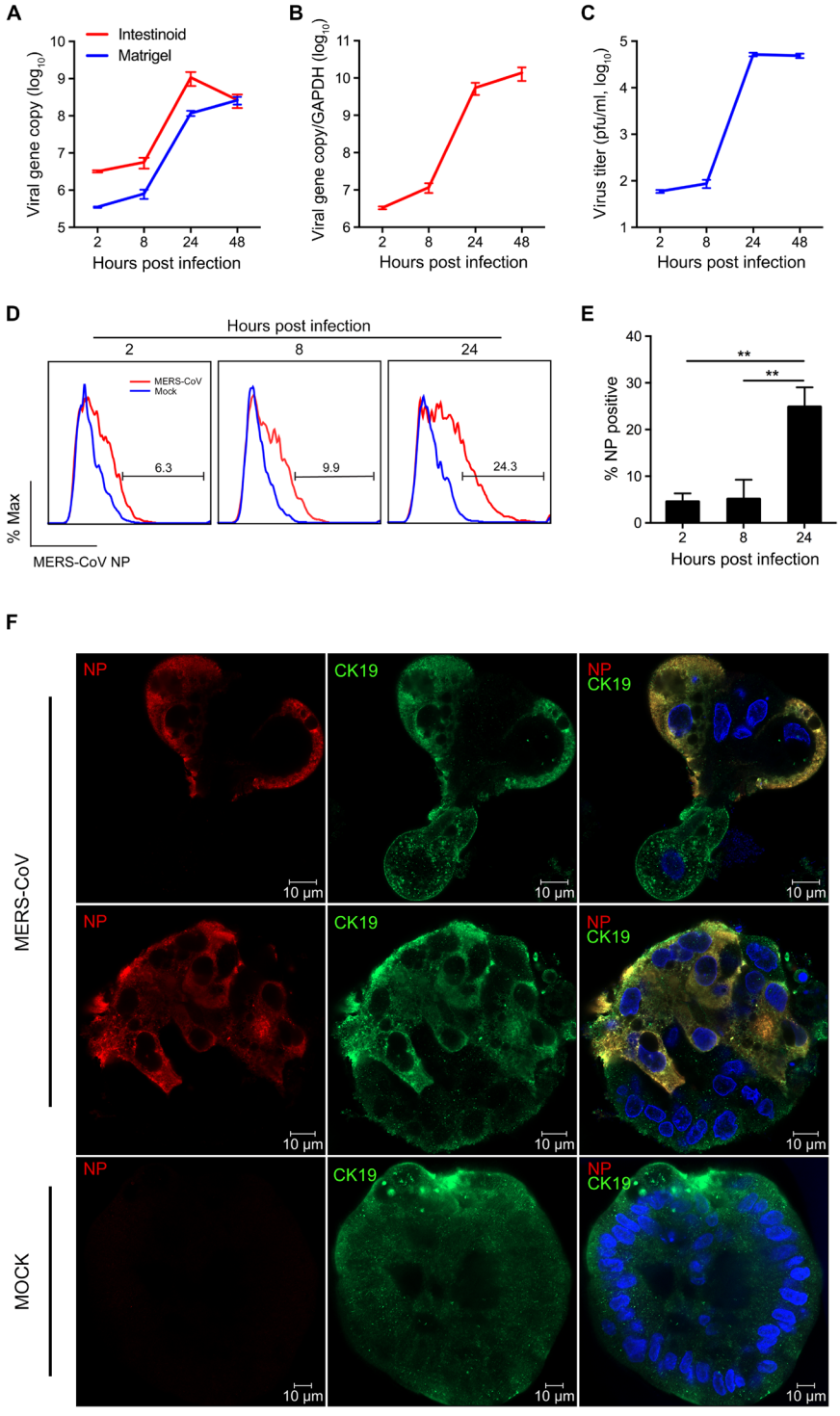


Fig. 2. MERS-CoV infection and replication in human intestinal organoids. The differentiated intestinoids were inoculated with MERS-CoV (MOI ≈ 0.05) in duplicate and then re-embedded in Matrigel and maintained in culture medium. (**A**) At the indicated hpi, intestinoids (Intestinoid), cell-free Matrigel, and culture medium (Matrigel) were harvested for the quantification of viral load with RT-qPCR. Serially diluted MERS-CoV NP plasmids were used to generate a standard curve for the quantification. (**B**) The absolute viral loads in intestinoids were normalized with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA transcripts. (**C**) The dissolved Matrigel and culture medium were applied to viral titration with plaque assay. Data are means ± SD of one representative experiment independently repeated three times. (**D** and **E**) At 24 hpi, the infected and mock-infected intestinoids were fixed after disassociation and stained with the MERS-CoV NP antiserum and then applied to flow cytometry to evaluate the percentage of NP-positive cells. The histogram shows the results of one representative experiment. (E) Data are means and SD of three independent experiments. Student's *t* test was used for data analysis. $^{**}P \leq 0.01$. (**F**) At 24 hpi, the infected (MERS-CoV) and mock-infected intestinoids, after fixation and immunofluorescence staining of MERS-CoV NP and CK19, were whole-mounted and imaged with a confocal microscope.

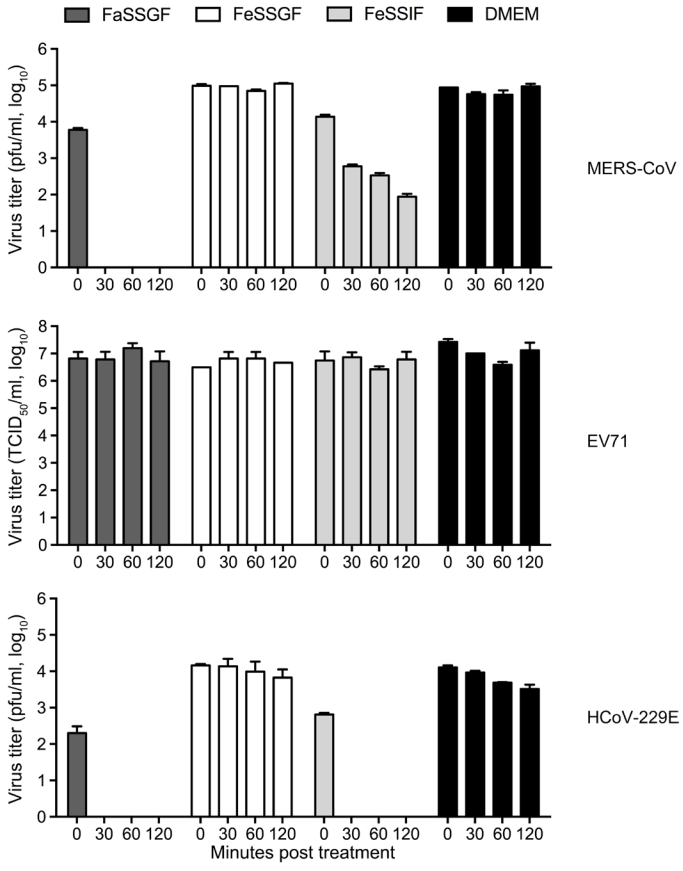


Fig. 3. MERS-CoV treatment with simulated gastrointestinal fluids. MERS-CoV solution [7.5×10^6 plaque-forming units (PFU), 500 μl] was mixed with 5 ml of FaSSGF, FeSSGF, FeSSIF, or DMEM and then incubated at 37°C for the indicated minutes. An aliquot (1 ml) of the virus/fluid mixture was sampled for virus titration in Vero-E6 cells in triplicate after neutralization with sodium hydroxide. The enterovirus EV71 and the human coronavirus hCoV-229E were treated with the same gastrointestinal fluids and titrated with $TCID_{50}$ (median tissue culture infectious dose) assay in RD cells and plaque assay in Huh7 cells, respectively. Data are from a representative experiment independently performed three times.

MERS-CoV infection and virus release in polarized intestinal epithelial cells

Caco-2 cells cultured in Transwell inserts can undergo spontaneous differentiation, display morphological and functional features of enterocytes, and form an intact permeability barrier, which could be reflected by heightened transepithelial electronic resistance (TEER), blockage of fluorescent molecule penetration, and formation of cell adhesion architecture (*22*). Thus, polarized Caco-2 cells in Transwell culture have been used to model human gastrointestinal epithelium and cellular barrier across the epithelium (*22*). We used the polarized Caco-2 cells to recapitulate the kinetics of viral growth and pattern of virus release in human intestinal epithelium after MERS-CoV inoculation.

After 2 weeks of Transwell culture, the polarized Caco-2 cells formed an intact epithelial barrier, as shown in fig. S3. The cell monolayers were then apically or basolaterally inoculated with MERS-CoV at an MOI of 0.1. Cell-free media in the upper and bottom chambers were harvested at the indicated time points for viral load quantification. Because of the formation of an intact epithelial barrier in the polarized Caco-2 cells, the viral loads detected from the media of the upper and bottom chambers can reflect the genuine pattern of virus release. As shown in Fig. 4A, after both apical and basolateral inoculation, the virus significantly replicated and bilaterally released into the media in the upper and bottom chambers. No infectious virus was detectable in the culture media at 2 hpi, whereas progeny virions were released from the apical and basolateral sides in both inoculation routes at 24 hpi (Fig. 4B). In addition, apical inoculation was more effective in establishing infection than basolateral inoculation. At 24 hours after a high MOI (2.0) inoculation, the infection rate, that is, the percentage of MERS-CoV NP–positive cells in apical inoculation, was significantly higher than that in basolateral inoculation ($P = 0.038$, Fig. 4C). Accordingly, the active caspase-3–positive cells, which underwent virus-induced apoptosis, were significantly more abundant in apical inoculation than in basolateral inoculation ($P = 0.001$). The higher infection efficiency in apical inoculation was further substantiated in immunofluorescence staining, which markedly revealed more abundant NP-positive cells via apical inoculation than basolateral inoculation (Fig. 4D). Collectively, both apical and basolateral inoculations of MERS-CoV resulted in robust viral replication in the polarized Caco-2 cells. Progeny virions were released bilaterally upon infection. Moreover, apical MERS-CoV inoculation was more effective in establishing infection than basolateral inoculation.

MERS-CoV infection in hDPP4 transgenic mice

We proceeded to evaluate whether direct intragastric MERS-CoV inoculation in the hDPP4 transgenic mouse can cause gastrointestinal infection and whether the gastrointestinal infection is followed by a respiratory infection. The hDPP4 mouse experiments were performed using our previously established mouse model of MERS-CoV infection (*23*). First, nine female mice were inoculated with 10^5 PFU of MERS-CoV via intragastric gavage; three of them were pretreated with a proton pump inhibitor, pantoprazole, to improve the viability of MERS-CoV in the mouse stomach because the in vitro experiment suggested the acid lability of MERS-CoV. At day 5 after virus inoculation, three pantoprazole-pretreated mice and three phosphate-buffered saline (PBS)–pretreated mice were sacrificed. At day 8 after inoculation, two mice lost more than 10% of their body weight and were sacrificed, whereas one mouse succumbed to the infection. Thus, direct intragastric MERS-CoV inoculation in hDPP4 mice may result in a lethal infection. To characterize the early events after intragastric MERS-CoV inoculation, nine female hDPP4 mice were inoculated via intragastric gavage; three mice were sacrificed at days 1, 3, and 5 after challenge, respectively. In addition, to exclude possible virus access to the airway during intragastric gavage, we directly injected 10^5 PFU of MERS-CoV into the stomach (intragastric injection hereafter) of 12 hDPP4 mice after a minor laparotomy. Six mice were pretreated with pantoprazole or mock-treated with PBS before the inoculation; three mice of each group were sacrificed at days 3 and 5 after inoculation.

Histopathological examination revealed that small intestines of all intragastrically inoculated mice displayed increased mononuclear cell infiltration in the lamina propria, with broadening of the intestinal villi and increased sloughing of surface epithelium. The intervening mucosa was basically normal. Figure 5A (a to c) shows the pathology of small intestines in the mice sacrificed at days 1, 3, and 5 after inoculation, respectively, indicating deteriorating inflammation and epithelial degeneration after virus inoculation. The pantoprazole-treated mice sacrificed at day 5 displayed more extensive and more prominent pathology in small intestine than the PBS-treated mice (Fig. 5A, d and e).

The mock-infected mouse showed a negligible pathological change in the intestines (Fig. 5A, f). Immunofluorescence staining was performed to identify the viral antigen (NP)–positive cell in the intestines of the inoculated mice. It was shown that intestinal epithelial cells were intensively infected at day 1 after inoculation (Fig. 5B, a to c). The infected cells were patchily distributed in the surface epithelia of small intestines (Fig. 5B, a and b). In some areas, the infected enterocytes formed syncytia (Fig. 5B, c, and fig. S4, A and B). At days 5 and 8 after inoculation, the NP-positive cells were distributed more widely in small intestines and emerged in the lamina propria (arrows in Fig. 5B, d to f) and Peyer's patch (arrowhead in Fig. 5B, d). Double staining of viral NP and CD68 revealed that some infected cells in the lamina propria were macrophages (Fig. 5B, f). Consistent with the massive inflammatory infiltration in small intestines of the inoculated mice as shown by hematoxylin and eosin (H&E) staining, CD3-positive lymphocytes were more abundant in the infected mice (Fig. 5B, a, b, and e) than those in the mock-infected mouse (Fig. 5B, h). Although there was no overt pathology in colon epithelium, NP-positive epithelial cells were nevertheless sparsely distributed in colon epithelium of infected mice (Fig. 5B, g). The viral load increased in small intestines of intragastrically injected mice, especially in the pantoprazole-pretreated mice (Fig. 5C), indicating a correlation between viral replication and intestinal pathology. However, the attempt to identify live virus from the intestine failed because the inoculation of the intestine homogenates, even the diluted homogenates, caused significant cell death and detachment of the monolayers.

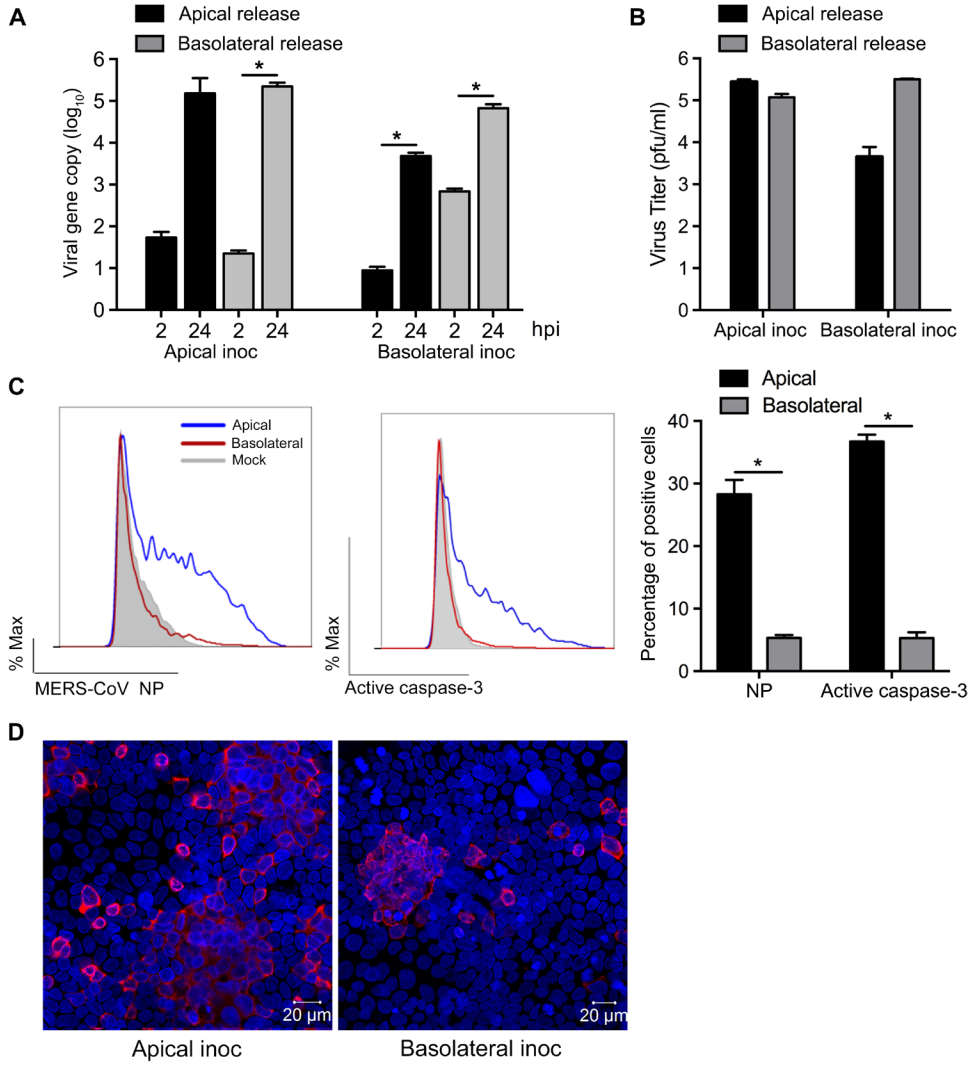


Fig. 4. MERS-CoV replication and cell apoptosis in the polarized Caco-2 cells. (**A** and **B**) The polarized Caco-2 cells were inoculated with MERS-CoV in duplicate from the apical or basolateral side of the monolayer with an MOI of 0.1. At the indicated hpi, cell-free media were harvested from the upper and bottom chambers for viral load quantification. The experiments were independently performed twice. A representative experiment is presented. (A) Viral gene copy number in the medium collected from the upper chamber (Apical release) and bottom chamber (Basolateral release) after apical inoculation (Apical inoc) and basolateral inoculation (Basolateral inoc). (B) Viral titer in the media harvested at 24 hpi detected by plaque assay. No plaque was detected in the media at 2 hpi. (**C** and **D**) The polarized Caco-2 cells were inoculated from the apical or basolateral side with MERS-CoV at an MOI of 2 or mock-inoculated. (C) At 24 hpi, cells were fixed after dissociation and applied to flow cytometry to detect the expression of MERS-CoV NP and activated caspase-3. The left and middle panels are the histograms of one representative experiment. The right panel presents means and SD of three independent experiments. *$P \leq 0.05$. (D) At 24 hpi, the polarized Caco-2 cells seeded on polycarbonate membranes were fixed and applied to immunofluorescence staining of MERS-CoV NP (red) and imaged en face.

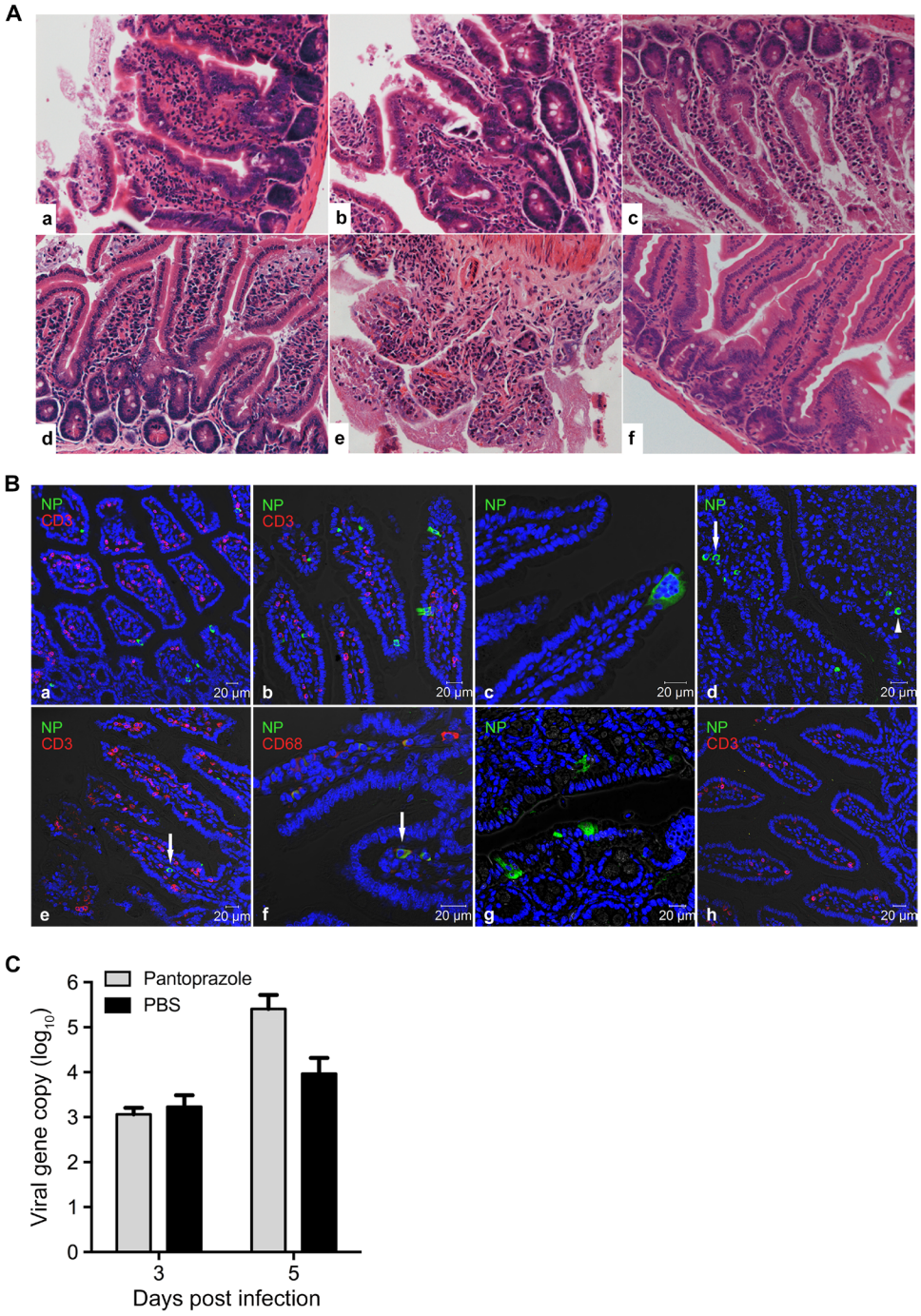


Fig. 5. MERS-CoV enteric infection in hDPP4 transgenic mice. hDPP4 mice were inoculated with MERS-CoV via direct intragastric gavage or intragastrically injected as described in Materials and Methods. (**A**) (a to c) Representative histopathology in small intestines at days 1, 3, and 5 after inoculation, respectively. (d and e) Small intestines of pantoprazole-pretreated mice at day 5 after inoculation. (f) Small intestine of a mock-infected mouse. H&E staining, magnification ×200. (**B**) Identification of MERS-CoV–infected cells in the intestines of the inoculated hDPP4 mice with immunostaining of NP and cell type markers. (a to c) Virus-positive enterocytes in mice at day 1 after inoculation. The infected enterocytes (c) form syncytia. (d to f) Virus-infected cells in mouse intestines at days 5 and 8 after inoculation, respectively. Arrows show NP-positive cells in the lamina propria, including the NP/CD68 double-positive macrophage (f). The arrowhead (in d) indicates virus-positive cells in Peyer's patch. (g) Virus-positive cells in the colon of an inoculated mouse. (h) Costaining of NP/CD3 in the mock-infected mouse. (**C**) Three intragastrically injected mice of the indicated groups were sacrificed at the indicated day after inoculation; intestine homogenates were applied for the quantification of viral load by RT-qPCR. The gray and black bars represent the viral loads of the mice pretreated with pantoprazole and mock-treated with PBS, respectively. Data are means and SD of three mice.

In the early phase up to day 5 after virus inoculation, the lung tissues of the inoculated mice showed minimal degree of inflammation (Fig. 6A, a). However, the pantoprazole-pretreated mice, which were sacrificed at the same day after inoculation, exhibited prominent pulmonary inflammation (Fig. 6A, b and c). Histological examination revealed marked infiltration of mononuclear cells and lymphocytes in the alveolar septa, indicating interstitial inflammation. Without pantoprazole pretreatment, the mice also exhibited inflammatory infiltration in the lung tissue at day 8 after intragastric gavage (Fig. 6A, d). Notably, NP-positive pneumocytes were identified in these mice (Fig. 6A, f). As expected, the abundance and distribution of virus-positive cells in the lung tissues of intragastrically inoculated mice were more limited than those in the intranasally inoculated mouse (fig. S4C). Consistent with the viral propagation in small intestines of intragastrically injected mice, viral load was elevated in the lung tissues of these mice at day 5 after inoculation, particularly in the pantoprazole-pretreated mice (Fig. 6B). Notably, infectious virions were detected in the lung homogenates of the intragastrically injected mice at day 5 after inoculation by plaque assay (fig. S5), which further verified the lung infection in these mice (Fig. 6A). We and others (*24*, *25*) observed brain infection in the intranasally inoculated hDPP4 mice. Likewise, the intragastrically injected mice also developed brain infection. Infectious virions were detected in the brain homogenates at day 5 after infection (fig. S5). The infected mice exhibited increased viral loads in the brain tissues. In summary, the direct intragastric MERS-CoV inoculation initiated an infection in the intestinal mucosa, leading to progressive inflammation and epithelial degeneration. With the progression of intestinal MERS-CoV infection, a sequential respiratory infection occurred.

DISCUSSION

The MERS epidemic has persisted for about 5 years. However, important issues related to the public health, such as mode of virus transmission and infection route, remain poorly understood. Because MERS primarily manifests as a respiratory infection, airway exposure is intuitively assumed to be the infection route, which contributes substantially to human MERS infections (*3*, *26*). Epidemiological studies, biological evaluation of the virus, and bioinformatic prediction collectively suggested that humans may also acquire MERS-CoV infection via the gastrointestinal tract. Here, we aimed to elucidate whether the gastrointestinal tract could serve as an alternative route to acquire MERS-CoV infection

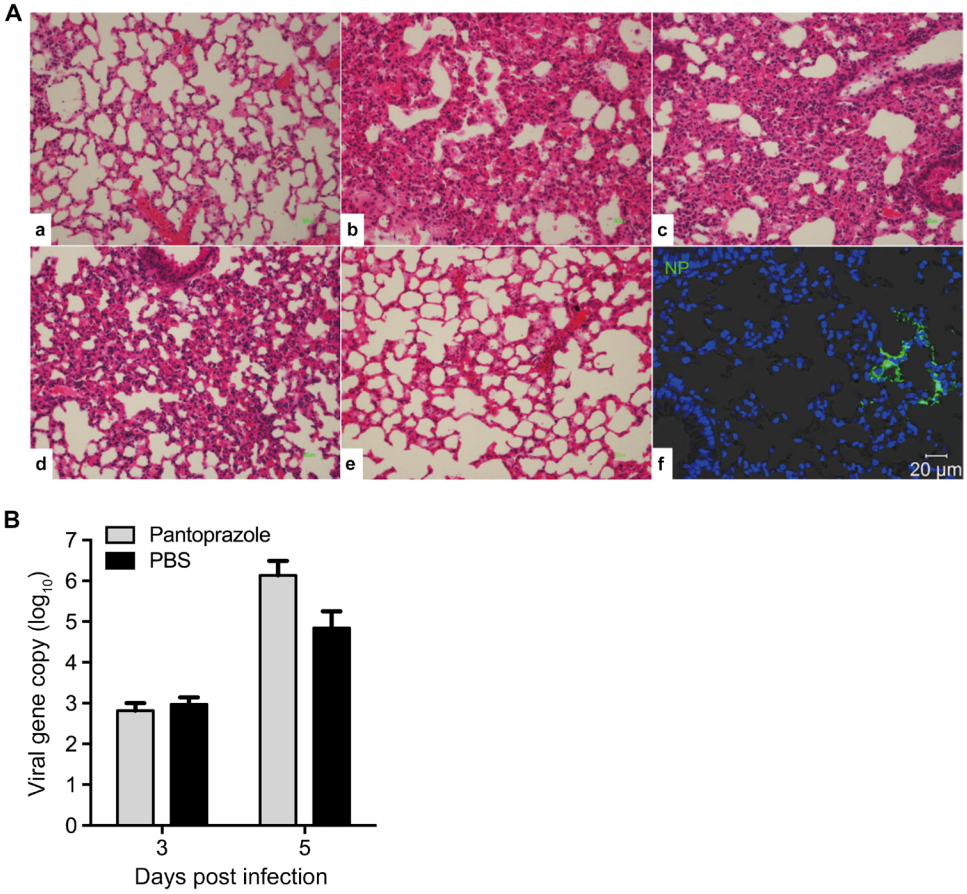


Fig. 6. Inflammation and MERS-CoV infection in the lung tissues of hDPP4 mice. (**A**) Lung tissues in the mice without pantoprazole pretreatment (a) versus those in pantoprazole pretreatment (b and c) at day 5 after intragastric MERS-CoV inoculation. (d) Lung pathology in an inoculated mouse at day 8 after intragastric inoculation without pantoprazole pretreatment. (f) Lung tissue of a mock-infected mouse. H&E staining, magnification ×200. (e) Immunostaining shows virus-positive cells (green) in the lung tissue of a mouse at day 8 after intragastric inoculation. (**B**) Lung tissues were harvested from three intragastrically injected mice at the indicated day after inoculation and homogenized for the quantification of viral load by RT-qPCR. The gray and black bars represent the viral loads of the mice pretreated with pantoprazole and mock-treated with PBS, respectively. Data are means and SD of three mice.

and whether gastrointestinal tract exposure to MERS-CoV may eventually lead to a respiratory infection.

We demonstrated that human primary intestinal epithelial cells were highly susceptible to MERS-CoV and could support viral replication (Fig. 1A). MERS-CoV infection and replication were further verified in an ex vivo culture of human small intestine (Fig. 1, B and C) and reinforced by identification of the viral replication intermediate in the stool specimen of a MERS patient (Fig. 1D). We further characterized MERS-CoV infection and replication in human intestinoids (Fig. 2), a novel ex vivo model system that can faithfully simulate the in vivo human intestinal epithelium. Notably, MERS-CoV replicated more robustly in human intestinoids than in primary respiratory epithelial cells and ex vivo lung tissues (*27*, *28*). The MERS-CoV receptor DPP4 is known as the major determinant of host and tissue tropisms in MERS-CoV (*29*). According to the public database Human Protein Atlas, human small intestine expresses the highest level of DPP4 mRNA and protein among all human organs, including lung and bronchus. Thus, the abundantly expressed DPP4 in human intestine may account for high susceptibility of these cells to MERS-CoV.

We also showed that MERS-CoV lost infectivity in highly acidic fasted-state gastric fluid, whereas it was considerably resistant to fed-state gastric fluid and intestinal fluid, which contains a large amount of digestive enzymes and bile salts (Fig. 3). It was documented that enveloped coronaviruses, unlike most enveloped viruses, are notably resistant to bile salts (*30*). Here, we verified the stability of MERS-CoV in bile salts in a very stringent setting, where the viruses were incubated with 10 volumes of FeSSIF containing a high concentration of bile salts. However, the acid instability of MERS-CoV shown in Fig. 3 seemed paradoxical, now that the intragastric inoculation of MERS-CoV caused the enteric infection in hDPP4 mice. Coincidently, an earlier study with a similar experimental setting documented the acid lability of rotavirus (*31*), a typical gastrointestinal virus that commonly causes gastroenteritis in mammals. In our study, apart from the same pH of 2.0 as in the previous study, pepsin was additionally supplemented to better simulate the authentic fasted-state gastric fluid. Thus, the lability of MERS-CoV in highly acidic FaSSGF, as shown in the in vitro assay, was probably an exaggerated result. In a real-life scenario, especially during food intake, the virus may not be exposed to such a large volume of pure gastrointestinal fluids as in the experimental setting.

MERS-CoV intestinal infection was hinted in an earlier study of intranasally inoculated hDPP4 mice (*24*), in which an increasing viral load was observed in mouse intestines after MERS-CoV inoculation. Here, we characterized MERS-CoV enteric infection in hDPP4 transgenic mice. After intragastric inoculation, the hDPP4 mice displayed viral antigen (NP)–positive cells, deteriorating inflammation, and epithelial degeneration in small intestines (Fig. 5, A and B). The viral NP-positive cells, which were distributed focally at day 1 after inoculation, became more diffuse in intestinal mucosa over time (Fig. 5B). In Transwell culture, the infected Caco-2 cells can release progeny virions apically and basolaterally (Fig. 4). Thus, it was not unexpected that NP-positive cells appeared in the lamina propria and lymphoid tissue of small intestine as the enteric infection progressed (Fig. 5B). Likewise, it is conceivable that the viruses produced in the intestinal mucosa of hDPP4 mouse may access the lymph flow and/or the bloodstream via the abundant lymphatic vessels and venules in the lamina propria of small intestine, respectively; they could be further transported to right heart via the thoracic duct and superior vena cava, and are disseminated to the lungs. The intragastrically inoculated mice exhibited minimal lung pathology at the early phase. Interstitial pneumonia occurred as the enteric infection developed; meanwhile, NP-positive cells emerged in the pulmonary parenchyma (Fig. 6A). Correspondingly, live viruses were identified in the lung homogenates of intragastrically injected mice, in which aspiration of the virus inoculum into airway was basically excluded. Consistent with the possibility of hematogenous viral trafficking, the lung tissues of intragastrically inoculated mice showed prominent interstitial pneumonia with thickened alveolar septa and mononuclear cell filtration. As frequently reported in the intranasally inoculated hDPP4 mice, brain infection also occurred in our intragastrically infected mice (fig. S5). Thus, further investigation is required to exclude the possibility that the dysfunctional brainstem due to brain infection may be attributable to the lung infection. Nevertheless, our results indicated that the MERS-CoV pulmonary infection in hDPP4 mice was secondary to the intestinal infection. Clinically, respiratory symptoms may not be the initial presentations of MERS patients (*32*, *33*). Respiratory infection as a subsequent manifestation was documented in some MERS patients, whose onset of symptoms was diarrhea and fever. In a case report, lung inflammation was an incidental finding on chest radiograph. The patient subsequently developed severe respiratory disease and died (*34*).

Virus dissemination and infection of other organs after gastrointestinal tract exposure occur in other viruses. For example, the direct intragastric inoculation of the avian influenza virus H5N1 in ferrets and hamsters caused systemic viral dissemination via the bloodstream (*35*). Human picornavirus, hepatitis A virus, enters the bloodstream after infecting human intestinal epithelium and ultimately establishes infection in the liver (*36*). Apart from the respiratory tract, the human alimentary tract is another common site for viral entry. Enteric viruses and even some non-enteric viruses can bypass the physical barriers and infect susceptible cells in the alimentary tract. To date, six human coronaviruses have been identified as the causative agents of mild or severe respiratory infections. The concomitant gastrointestinal symptoms are frequently observed in some human coronaviruses. For example, the human coronavirus OC43, which is closely related to bovine coronavirus phylogenetically, caused gastrointestinal symptoms in up to 57% of the infected patients (*37*). Notably, enteric involvement was verified in human infection of severe acute respiratory syndrome coronavirus (SARS-CoV), which caused a large-scale epidemic with more than 8000 human infections in 2002–2003 (*38*). Active SARS-CoV replication was identified in the small and large intestine specimens from colonoscopy biopsy and autopsy (*39*). A significant portion of SARS patients had gastrointestinal symptoms; the viral load was the highest in stool specimens, followed by nasopharyngeal aspiration specimens (*40*). Thus, the human gastrointestinal tract was speculated to be the primary infection site of SARS-CoV (*41*), which raised a fearsome concern of dual infectivity of SARS-CoV as being both a respiratory and gastrointestinal pathogen. However, no serious study has been reported to address the important issue. In contrast to the ambiguity of virus acquisition via the alimentary tract in human coronaviruses, it has been known that most animal coronaviruses, such as bovine coronavirus and porcine transmissible gastroenteritis virus, can primarily infect either the enteric or respiratory tract or sometimes translocate between sites (*42*, *43*). Until now, however, whether human and animal coronaviruses share similarity in terms of a dual acquisition route has remained unanswered. Here, we addressed this long-standing question in human intestinal epithelial cells, intestinal organoids, and hDPP4 transgenic mice. We demonstrate that the human intestinal tract serves as an alternative infection route for MERS-CoV. The knowledge obtained from this study will provide a scientific basis for the implementation

of effective intervention/prevention strategies to halt the continuing MERS epidemic.

MATERIALS AND METHODS

Virus infection in human primary intestinal epithelial cells, small intestine explants, intestinal organoids, and polarized Caco-2 cells

MERS-CoV of the EMC/2012 strain was provided by R. Fouchier (Erasmus Medical Center) (*1*). Clonetics normal human intestinal epithelial cells (InEpC, Lonza) were cultured in SmGM-2 Bullet medium with growth supplements. At 24 hours after MERS-CoV inoculation with an MOI of about 10, cells seeded on chamber slides were fixed with 4% paraformaldehyde and applied to immunofluorescence staining; cell-free medium and cells in a 96-well plate were harvested for the quantification of viral load with viral RNA extraction and RT-qPCR, as we described previously (*44*). Under the protocol approved by Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB, UW 13-364), normal small intestine was obtained for virus inoculation from a patient who underwent surgical resection. The full-thickness tissue was excised into about 0.3 cm × 0.6 cm strips, positioned in a 12-well culture plate, and incubated with 10^7 PFU of MERS-CoV at 37°C for 2 hours. After the inoculated explants were rinsed with MEM supplemented with antibiotics, they were positioned onto Transwell inserts precoated with Matrigel. More Matrigel was applied to brace around the explants, with mucosa facing upward. The bottom chamber contained 1 ml of DMEM supplemented with six antibiotics (detailed information will be provided upon request). At the indicated hpi, the explants were harvested for the quantification of viral load and immunofluorescence staining after fixation.

Intestinal organoids derived from normal human colon were cultured, and differentiation was induced as described elsewhere (*18*). The differentiated intestinoids were mechanically sheared with Pasteur pipettes and incubated with MERS-CoV at 37°C for 2 hours with the addition of 10 μM Rho-associated coiled-coil–containing protein kinase (ROCK) inhibitor Y-27632 (STEMCELL Technologies) to inhibit spontaneous apoptosis during the inoculation. An inoculum of 10^5 PFU of MERS-CoV was used to infect one droplet of intestinoids (containing 50 to 100 intestinoids), with an estimated MOI of 0.05. After the inoculum was removed, the virus-inoculated intestinoids were rinsed with PBS and then re-embedded in Matrigel and cultured in a 24-well plate with culture medium. At the indicated hpi, the intestinoids were harvested for the quantification of viral load with RNA extraction and RT-qPCR, whereas cell-free Matrigel and culture medium were used for viral load detection and viral titration with plaque assay. In addition, infected or mock-infected intestinoids were fixed with 4% paraformaldehyde at 24 hpi, followed by immunofluorescence staining and confocal imaging. Moreover, a proportion of infected or mock-infected intestinoids, after fixation and antibody staining, were applied to flow cytometry for the examination of infection rate.

Caco-2 cells were seeded on polycarbonate inserts (0.4 μm pore size) of a 12-well Transwell plate (Corning) at a cell density of 10^5 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum (FBS). Culture medium was changed every 3 days. The TEER was measured every 3 days using Millicell ERS-2 Volt-Ohm Meter (EMD Millipore). To monitor the integrity of the Caco-2 monolayer as an epithelial barrier, at the indicated day after seeding, fluorescein isothiocyanate–dextran with a molecular weight of 10 k (Sigma-Aldrich) was added in the culture medium of the upper chamber at a concentration of 1 mg/ml and incubated at 37°C for 4 hours. Subsequently, the culture media were harvested from the upper and bottom chambers to detect the fluorescence intensity using the Victor XIII Multilabel Reader (PerkinElmer). When TEER and epithelial integrity reached a plateau after 2 weeks of Transwell culture, the polarized Caco-2 cells were inoculated with MERS-CoV from the apical or basolateral side of the monolayer at an MOI of 0.1 and then maintained in DMEM without FBS. At the indicated time points after inoculation, cell-free media were harvested from the upper and bottom chambers for viral load quantification and viral titration. To examine the MERS-CoV infection efficiency in the polarized Caco-2 cells, the cells were inoculated from the apical or basolateral side with MERS-CoV at an MOI of 2 or mock-inoculated. At 24 hpi, Caco-2 monolayers were disassociated into a single-cell suspension after incubation with 0.25% trypsin-EDTA for 30 min and then fixed for flow cytometry analysis after antibody staining. The polarized Caco-2 cells seeded on polycarbonate membranes were fixed and then excited from Transwell inserts for immunofluorescence staining.

Detection of subgenomic RNA in stool specimens of MERS patients

We applied RT-PCR assays, using a leader-specific primer, and primer and probe targeting sequence downstream of the start codon of the most abundant N gene sgRNA to test whether sgRNA, the replication intermediate, is detectable in MERS-CoV RNA–positive stool specimens collected from MERS patients (*17*). Oligonucleotide sequences are as follows: MERS-sgRNA-rtF, ACTTCCCCTCGTTCTCTTGCAG; MERS-sgRNA-rtP, FAM-CACGAGCTGCACCAAATAACACTGTCTC-BHQ; and MERS-sgRNA-rtR, GTAAGAGGGACTTTCCCGTGTTG. SSIII RT-PCR kit (Thermo Fisher Scientific) was used with 400 nM of each of the primers and 200 nM of the probe. Thermal cycling involved 10 min at 55°C for reverse transcription, followed by 3 min at 95°C and 45 cycles of 10 s at 95°C, 10 s at 56°C, and 20 s at 72°C. The products of sgRNA RT-PCR assay were analyzed on agarose gel, and tentative bands were sequenced.

MERS-CoV treatment with simulated gastrointestinal fluids

FaSSGF, FeSSGF, and FeSSIF powders (Biorelevant) were prepared into solutions according to the manufacturer's instruction. FaSSGF was additionally supplemented with pepsin (0.1 mg/ml; Sigma-Aldrich). A volume of 500 μl of MERS-CoV solution (7.5×10^6 PFU) was mixed with 5 ml of FaSSGF, FeSSIF, FeSSGF, or DMEM and then incubated at 37°C for the indicated minutes. An aliquot (1 ml) of the virus/fluid mixture was then sampled for virus titration in Vero-E6 cells after neutralization to pH 7.0 with 2.5 M sodium hydroxide. Human enterovirus EV71 and human coronavirus hCoV-229E were treated with the stimulated gastrointestinal fluids for comparison.

MERS-CoV infection in hDPP4 transgenic mice

First, nine female hDPP4 mice, 8 to 9 weeks old, were inoculated with 10^5 PFU of MERS-CoV (200 μl) via direct intragastric gavage. Three mice were administered with the proton pump inhibitor pantoprazole (40 mg/kg body weight) or PBS via intraperitoneal injection 2 hours before the virus challenge. At day 5 after virus inoculation, three pantoprazole-treated and three PBS-treated mice were sacrificed to harvest the lung and intestine for histopathological examination and viral load detection. Next, nine female hDPP4 mice were infected via intragastric gavage to characterize the early events after MERS-CoV inoculation. At days 1, 3, and 5 after intragastric gavage of MERS-CoV,

three mice were sacrificed to harvest the lung and intestine. To exclude the possible aspiration to the mouse airway during the intragastric gavage, 12 female mice, 6 of them pretreated with pantoprazole or mock-treated with PBS, were directly injected with 10^5 PFU of MERS-CoV (100 μl) into the mouse stomach after a 0.5-cm incision was made in the abdominal wall to expose the mouse stomach. Three pantoprazole- and PBS-treated mice were sacrificed at days 3 and 5 after infection. Mouse small intestine, lung, and brain were homogenized to detect live virus with plaque assay and quantify viral load with RT-qPCR. In each experiment, at least one mouse was inoculated with PBS as the mock infection control. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology (BIME 2015-0025).

Immunofluorescence staining of the infected cells, intestinoids, and tissues

To identify virus-positive cells, cells on chamber slides and Transwell inserts, human intestinoids, slides of human small intestine, or mouse tissues were subjected to immunofluorescence staining, as described previously (*45*). Briefly, after permeabilization and blocking, cells, intestinoids, and tissues were stained with a guinea pig antiserum against MERS-CoV NP, followed with secondary antibodies including goat anti–guinea pig Alexa Fluor 488 immunoglobulin G (IgG) or goat anti–guinea pig Alexa Fluor 594 IgG (Abcam). For intestine explants and intestinoids, CK19 (YM3051, ImmunoWay), a marker of intestinal epithelial cell, was costained with an anti-NP antibody. To define the identity of the viral NP-positive cells in the tissues of infected hDPP4 mice, apart from the labeling of MERS-CoV NP, double staining was performed using a rat anti-mouse monoclonal CD68 antibody (FA-11, Abcam) and Alexa Fluor 568 goat anti-rat IgG (Life Technologies). After staining, intestinoids were whole-mounted with VECTASHIELD HardSet Antifade Mounting Medium (Vector Laboratories). The whole-mounted intestinoids, chamber slides, Transwell inserts, and tissue slides were imaged using a Carl Zeiss LSM 780 or 800 confocal microscope.

Flow cytometry analysis

MERS-CoV–infected human intestinoids and Transwell-cultured Caco-2 cells were digested into a single-cell suspension and fixed with 4% paraformaldehyde. MERS-CoV–positive cell was detected with the same MERS-CoV NP antiserum and goat anti–guinea pig Alexa Fluor 488 IgG, as described above. Active caspase-3 was detected with an Alexa Fluor 647–conjugated rabbit anti-human active caspase-3 antibody (BD Pharmingen). Cell permeabilization for intracellular staining was performed with 0.1% Triton X-100 in PBS. Immunostaining for flow cytometry was performed following standard procedures (*46*). The flow cytometry was performed using the FACSCanto II Analyzer (BD Biosciences), and data were analyzed using FlowJo vX (Tree Star).

Statistical analysis

Student's *t* test was used for data analysis. $P < 0.05$ was considered to be statistically significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/11/eaao4966/DC1

fig. S1. MERS-CoV replication in human primary intestinal epithelial cells.

fig. S2. Expression of human DPP4 on the surface of epithelial cells in intestinoids.

fig. S3. Formation of intact epithelial barrier in the polarized Caco-2 cells after Transwell culture.

fig. S4. The virus-positive cells in small intestine of an intragastrically inoculated mouse and those in the lung tissue of an intranasally inoculated mouse.

fig. S5. Identification of live viruses in the lung and brain homogenates and increased viral load in the brain tissues of intragastrically injected mice.

REFERENCES AND NOTES

1. A. M. Zaki, S. van Boheemen, T. M. Bestebroer, A. D. M. E. Osterhaus, R. A. M. Fouchier, Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N. Engl. J. Med.* **367**, 1814–1820 (2012).
2. World Health Organization, *MERS-CoV Outbreak Update* (World Health Organization, 2017); www.who.int/emergencies/mers-cov/en/.
3. A. Assiri, J. A. Al-Tawfiq, A. A. Al-Rabeeah, F. A. Al-Rabiah, S. Al-Hajjar, A. Al-Barrak, H. Flemban, W. N. Al-Nassir, H. H. Balkhy, R. F. Al-Hakeem, H. Q. Makhdoom, A. I. Zumla, Z. A. Memish, Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: A descriptive study. *Lancet Infect. Dis.* **13**, 752–761 (2013).
4. J. F. W. Chan, S. K. P. Lau, K. K. W. To, V. C. C. Cheng, P. C. Y. Woo, K.-Y. Yuen, Middle East respiratory syndrome coronavirus: Another zoonotic betacoronavirus causing SARS-like disease. *Clin. Microbiol. Rev.* **28**, 465–522 (2015).
5. S. Cauchemez, P. Nouvellet, A. Cori, T. Jombart, T. Garske, H. Clapham, S. Moore, H. L. Mills, H. Salje, C. Collins, I. Rodriquez-Barraquer, S. Riley, S. Truelove, H. Algarni, R. Alhakeem, K. AlHarbi, A. Turkistani, R. J. Aguas, D. A. T. Cummings, M. D. Van Kerkhove, C. A. Donnelly, J. Lessler, C. Fraser, A. Al-Barrak, N. M. Ferguson, Unraveling the drivers of MERS-CoV transmission. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 9081–9086 (2016).
6. D. R. Adney, N. van Doremalen, V. R. Brown, T. Bushmaker, D. Scott, E. de Wit, R. A. Bowen, V. J. Munster, Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. *Emerg. Infect. Dis.* **20**, 1999–2005 (2014).
7. C. B. Reusken, E. A. Farag, M. Jonges, G. J. Godeke, A. M. El-Sayed, S. D. Pas, V. S. Raj, K. A. Mohran, H. A. Moussa, H. Ghobashy, F. Alhajri, A. K. Ibrahim, B. J. Bosch, S. K. Pasha, H. E. Al-Romaihi, M. Al-Thani, S. A. Al-Marri, M. M. AlHajri, B. L. Haagmans, M. P. Koopmans, Middle East respiratory syndrome coronavirus (MERS-CoV) RNA and neutralising antibodies in milk collected according to local customs from dromedary camels, Qatar, April 2014. *Euro. Surveill.* **19**, 20829 (2014).
8. N. van Doremalen, T. Bushmaker, W. B. Karesh, V. J. Munster, Stability of Middle East respiratory syndrome coronavirus in milk. *Emerg. Infect. Dis.* **20**, 1263–1264 (2014).
9. Z. A. Memish, M. Cotten, B. Meyer, S. J. Watson, A. J. Alsahafi, A. A. Al Rabeeah, V. M. Corman, A. Sieberg, H. Q. Makhdoom, A. Assiri, M. Al Masri, S. Aldabbagh, B.-J. Bosch, M. Beer, M. A. Müller, P. Kellam, C. Drosten, Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. *Emerg. Infect. Dis.* **20**, 1012–1015 (2014).
10. P. Durai, M. Batool, M. Shah, S. Choi, Middle East respiratory syndrome coronavirus: Transmission, virology and therapeutic targeting to aid in outbreak control. *Exp. Mol. Med.* **47**, e181 (2015).
11. J. A. Al-Tawfiq, Z. A. Memish, Middle East respiratory syndrome coronavirus: Epidemiology and disease control measures. *Infect. Drug Resist.* **7**, 281–287 (2014).
12. S. S. Lee, N. S. Wong, Probable transmission chains of Middle East respiratory syndrome coronavirus and the multiple generations of secondary infection in South Korea. *Int. J. Infect. Dis.* **38**, 65–67 (2015).
13. N. van Doremalen, T. Bushmaker, V. J. Munster, Stability of Middle East respiratory syndrome coronavirus (MERS-CoV) under different environmental conditions. *Euro. Surveill.* **18**, 20590 (2013).
14. S. Y. Bin, J. Y. Heo, M.-S. Song, J. Lee, E.-H. Kim, S. J. Park, H. I. Kwon, S. M. Kim, Y.-i. Kim, Y.-J. Si, I.-W. Lee, Y. H. Baek, W.-S. Choi, J. Min, H. W. Jeong, Y. K. Choi, Environmental contamination and viral shedding in MERS patients during MERS-CoV outbreak in South Korea. *Clin. Infect. Dis.* **62**, 755–760 (2016).
15. G. K.-M. Goh, A. K. Dunker, V. N. Uversky, Understanding viral transmission behavior via protein intrinsic disorder prediction: Coronaviruses. *J. Pathog.* **2012**, 738590 (2012).
16. G. K.-M. Goh, A. K. Dunker, V. Uversky, Prediction of intrinsic disorder in MERS-CoV/HCoV-EMC supports a high oral-fecal transmission. *PLOS Curr.* **5** (2013).
17. V. M. Corman, A. M. Albarrak, A. S. Omrani, M. M. Albarrak, M. E. Farah, M. Almasri, D. Muth, A. Sieberg, B. Meyer, A. M. Assiri, T. Binger, K. Steinhagen, E. Lattwein, J. Al-Tawfiq, M. A. Müller, C. Drosten, Z. A. Memish, Viral shedding and antibody response in 37 patients with Middle East respiratory syndrome coronavirus infection. *Clin. Infect. Dis.* **62**, 477–483 (2016).
18. T. Sato, D. E. Stange, M. Ferrante, R. G. Vries, J. H. Van Es, S. Van den Brink, W. J. Van Houdt, A. Pronk, J. Van Gorp, P. D. Siersema, H. Clevers, Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762–1772 (2011).

19. K. Wang, S. T. Yuen, J. Xu, S. P. Lee, H. H. N. Yan, S. T. Shi, H. C. Siu, S. Deng, K. M. Chu, S. Law, K. H. Chan, A. S. Chan, W. Y. Tsui, S. L. Ho, A. K. W. Chan, J. L. K. Man, V. Foglizzo, M. K. Ng, A. S. Chan, Y. P. Ching, G. H. W. Cheng, T. Xie, J. Fernandez, V. S. W. Li, H. Clevers, P. A. Rejto, M. Mao, S. Y. Leung, Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. *Nat. Genet.* **46**, 573–582 (2014).
20. Y. Yin, M. Bijvelds, W. Dang, L. Xu, A. A. van der Eijk, K. Knipping, N. Tuysuz, J. F. Dekkers, Y. Wang, J. de Jonge, D. Sprengers, L. J. van der Laan, J. M. Beekman, D. Ten Berge, H. J. Metselaar, H. de Jonge, M. P. Koopmans, M. P. Peppelenbosch, Q. Pan, Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. *Antiviral Res.* **123**, 120–131 (2015).
21. S. R. Finkbeiner, X.-L. Zeng, B. Utama, R. L. Atmar, N. F. Shroyer, M. K. Estes, Stem cell-derived human intestinal organoids as an infection model for rotaviruses. *MBio* **3**, e00159-12 (2012).
22. A. L. Kauffman, A. V. Gyurdieva, J. R. Mabus, C. Ferguson, Z. Yan, P. J. Hornby, Alternative functional in vitro models of human intestinal epithelia. *Front. Pharmacol.* **4**, 79 (2013).
23. G. Zhao, Y. Jiang, H. Qiu, T. Gao, Y. Zeng, Y. Guo, H. Yu, J. Li, Z. Kou, L. Du, W. Tan, S. Jiang, S. Sun, Y. Zhou, Multi-organ damage in human dipeptidyl peptidase 4 transgenic mice infected with Middle East respiratory syndrome-coronavirus. *PLOS ONE* **10**, e0145561 (2015).
24. X. Tao, T. Garron, A. S. Agrawal, A. Algaissi, B.-H. Peng, M. Wakamiya, T.-S. Chan, L. Lu, L. Du, S. Jiang, R. B. Couch, C.-T. Tseng, Characterization and demonstration of the value of a lethal mouse model of Middle East respiratory syndrome coronavirus infection and disease. *J. Virol.* **90**, 57–67 (2015).
25. K. Li, C. Wohlford-Lenane, S. Perlman, J. Zhao, A. K. Jewell, L. R. Reznikov, K. N. Gibson-Corley, D. K. Meyerholz, P. B. McCray Jr., Middle East respiratory syndrome coronavirus causes multiple organ damage and lethal disease in mice transgenic for human dipeptidyl peptidase 4. *J. Infect. Dis.* **213**, 712–722 (2016).
26. P. Gautret, G. C. Gray, R. N. Charrel, N. G. Odezulu, J. A. Al-Tawfiq, A. Zumla, Z. A. Memish, Emerging viral respiratory tract infections—Environmental risk factors and transmission. *Lancet Infect. Dis.* **14**, 1113–1122 (2014).
27. F. Zielecki, M. Weber, M. Eickmann, L. Spiegelberg, A. M. Zaki, M. Matrosovich, S. Becker, F. Weber, Human cell tropism and innate immune system interactions of human respiratory coronavirus EMC compared to those of severe acute respiratory syndrome coronavirus. *J. Virol.* **87**, 5300–5304 (2013).
28. R. W. Y. Chan, M. C. W. Chan, S. Agnihothram, L. L. Y. Chan, D. I. T. Kuok, J. H. M. Fong, Y. Guan, L. L. M. Poon, R. S. Baric, J. M. Nicholls, J. S. M. Peiris, Tropism of and innate immune responses to the novel human betacoronavirus lineage C virus in human ex vivo respiratory organ cultures. *J. Virol.* **87**, 6604–6614 (2013).
29. J. Zhou, H. Chu, J. F.-W. Chan, K.-Y. Yuen, Middle East respiratory syndrome coronavirus infection: Virus-host cell interactions and implications on pathogenesis. *Virol. J.* **12**, 218 (2015).
30. P. J. Quin, B. K. Markey, F. C. Leonard, E. S. Fitzpatrick, S. Fanning, P. Hartigan, *Veterinary Microbiology and Microbial Disease* (Wiley-Blackwell, ed. 2, 2011).
31. C. Weiss, H. F. Clark, Rapid inactivation of rotaviruses by exposure to acid buffer or acidic gastric juice. *J. Gen. Virol.* **66** (Pt. 12), 2725–2730 (1985).
32. A. Assiri, A. McGeer, T. M. Perl, C. S. Price, A. A. Al Rabeeah, D. A. T. Cummings, Z. N. Alabdullatif, M. Assad, A. Almulhim, H. Makhdoom, H. Madani, R. Alhakeem, J. A. Al-Tawfiq, M. Cotten, S. J. Watson, P. Kellam, A. I. Zumla, Z. A. Memish; KSA MERS-CoV Investigation Team, Hospital outbreak of Middle East respiratory syndrome coronavirus. *N. Engl. J. Med.* **369**, 407–416 (2013).
33. Z. A. Memish, A. I. Zumla, R. F. Al-Hakeem, A. A. Al-Rabeeah, G. M. Stephens, Family cluster of Middle East respiratory syndrome coronavirus infections. *N. Engl. J. Med.* **368**, 2487–2494 (2013).
34. A. Mailles, K. Blanckaert, P. Chaud, S. van der Werf, B. Lina, V. Caro, C. Campese, B. Guéry, H. Prouvost, X. Lemaire, M. C. Paty, S. Haeghebaert, D. Antoine, N. Ettahar, H. Noel, S. Behillil, S. Hendricx, J. C. Manuguerra, V. Enouf, G. La Ruche, C. Semaille, B. Coignard, D. Lévy-Bruhl, F. Weber, C. Saura, D. Che; Collective The investigation team, First cases of Middle East respiratory syndrome coronavirus (MERS-CoV) infections in France, investigations and implications for the prevention of human-to-human transmission, France, May 2013. *Euro. Surveill.* **18**, 20502 (2013).
35. K. Shinya, A. Makino, H. Tanaka, M. Hatta, T. Watanabe, M. Q. Le, H. Imai, Y. Kawaoka, Systemic dissemination of H5N1 influenza A viruses in ferrets and hamsters after direct intragastric inoculation. *J. Virol.* **85**, 4673–4678 (2011).
36. A. Martin, S. M. Lemon, Hepatitis A virus: From discovery to vaccines. *Hepatology* **43**, S164–S172 (2006).
37. A. Vabret, T. Mourez, S. Gouarin, J. Petitjean, F. Freymuth, An outbreak of coronavirus OC43 respiratory infection in Normandy, France. *Clin. Infect. Dis.* **36**, 985–989 (2003).
38. J. S. M. Peiris, S. T. Lai, L. L. M. Poon, Y. Guan, L. Y. C. Yam, W. Lim, J. Nicholls, W. K. S. Yee, W. W. Yan, M. T. Cheung, V. C. C. Cheng, K. H. Chan, D. N. C. Tsang, R. W. H. Yung, T. K. Ng, K. Y. Yuen: SARS study group, Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* **361**, 1319–1325 (2003).
39. W. K. Leung, K.-f. To, P. K. S. Chan, H. L. Y. Chan, A. K. L. Wu, N. Lee, K. Y. Yuen, J. J. Y. Sung, Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection. *Gastroenterology* **125**, 1011–1017 (2003).
40. I. F. N. Hung, V. C. C. Cheng, A. K. L. Wu, B. S. F. Tang, K. H. Chan, C. M. Chu, M. M. L. Wong, W. T. Hui, L. L. M. Poon, D. M. W. Tse, K. S. Chan, P. C. Y. Woo, S. K. P. Lau, J. S. M. Peiris, K. Y. Yuen, Viral loads in clinical specimens and SARS manifestations. *Emerg. Infect. Dis.* **10**, 1550–1557 (2004).
41. Y. Ding, L. He, Q. Zhang, Z. Huang, X. Che, J. Hou, H. Wang, H. Shen, L. Qiu, Z. Li, J. Geng, J. Cai, H. Han, X. Li, W. Kang, D. Weng, P. Liang, S. Jiang, Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: Implications for pathogenesis and virus transmission pathways. *J. Pathol.* **203**, 622–630 (2004).
42. P. J. Openshaw, Crossing barriers: Infections of the lung and the gut. *Mucosal Immunol.* **2**, 100–102 (2009).
43. L. J. Saif, Bovine respiratory coronavirus. *Vet. Clin. North Am. Food Anim. Pract.* **26**, 349–364 (2010).
44. J. Zhou, H. Chu, C. Li, B. H.-Y. Wong, Z.-S. Cheng, V. K.-M. Poon, T. Sun, C. C.-Y. Lau, K.-K. Wong, J. Y.-W. Chan, J. F.-W. Chan, K. K.-W. To, K.-H. Chan, B.-J. Zheng, K.-Y. Yuen, Active replication of Middle East respiratory syndrome coronavirus and aberrant induction of inflammatory cytokines and chemokines in human macrophages: Implications for pathogenesis. *J. Infect. Dis.* **209**, 1331–1342 (2014).
45. J. Zhou, T. Tan, Y. Tian, B. Zheng, J.-H. Ou, E. J. Huang, T. S. B. Yen, Krüppel-like factor 15 activates hepatitis B virus gene expression and replication. *Hepatology* **54**, 109–121 (2011).
46. H. Chu, J. Zhou, B. H.-Y. Wong, C. Li, J. F.-W. Chan, Z.-S. Cheng, D. Yang, D. Wang, A. C.-Y. Lee, C. Li, M.-L. Yeung, J.-P. Cai, I. H.-Y. Chan, W.-K. Ho, K.-K. To, B.-J. Zheng, Y. Yao, C. Qin, K.-Y. Yuen, Middle East respiratory syndrome coronavirus efficiently infects human primary T lymphocytes and activates the extrinsic and intrinsic apoptosis pathways. *J. Infect. Dis.* **213**, 904–914 (2016).

Acknowledgments: We thank H. Clevers (Hubrecht Institute) for providing the reagents for organoid cultures. We also thank C. C. Y. Yip, J.-P. Cai, and Faculty Core Facility (Li Ka Shing Faculty of Medicine, University of Hong Kong) for technical assistance. **Funding:** The study is partly supported by the Health and Medical Research Fund (reference nos.14131392 and 15140762) of Food and Health Bureau; the National Natural Science Foundation of China/ Research Grants Council Joint Research Scheme (N_HKU728/14); the Collaborative Research Fund (C7011-15R) of the Research Grants Council; the Theme-Based Research Scheme (T11/707/15) of the Research Grants Council, Hong Kong Special Administrative Region; Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Ministry of Education of China; the National Project of Infectious Disease (2014ZX10004001004), Ministry of Science and Technology of China; the German Research Foundation (Deutsche Forschungsgemeinschaft grants DR772/12-1 and DR772/7-2); and the European Commission project PREPARE (contract number 602525). **Author contributions:** J.Z. and K.-Y.Y. designed the study. J.Z., C.L., H.C., D.W., V.K.-M.P., B.H.-Y.W., X.Z., M.C.C., D.Y., and Y.W. performed the in vitro experiments. H.H.-N.Y. and S.Y.L. provided the intestinal organoids. G.Z. and S.S. performed the animal experiments. J.Z., C.L., H.C., D.W., L.W., J.F.-W.C., K.K.-W.T., and I.F.-N.H. analyzed and interpreted the data. R.K.H.A.-Y. reviewed histopathology. I.H.-Y.C. provided the normal human intestine. Z.A.M., V.M.C., and C.D. examined the specimens of MERS patients. Y.Z. provided the hDPP4 mice. J.Z., H.C., J.F.-W.C., K.K.-W.T., and K.-Y.Y. wrote and revised the manuscript. **Competing interests:** S.Y.L. has received research sponsorship from Pfizer, Merck, and Servier. The sponsors were not involved in the study design, data collection, and analysis. The other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 27 July 2017
Accepted 20 October 2017
Published 15 November 2017
10.1126/sciadv.aao4966

Citation: J. Zhou, C. Li, G. Zhao, H. Chu, D. Wang, H. H.-N. Yan, V. K.-M. Poon, L. Wen, B. H.-Y. Wong, X. Zhao, M. C. Chiu, D. Yang, Y. Wang, R. K. H. Au-Yeung, I. H.-Y. Chan, S. Sun, J. F.-W. Chan, K. K.-W. To, Z. A. Memish, V. M. Corman, C. Drosten, I. F.-N. Hung, Y. Zhou, S. Y. Leung, K.-Y. Yuen, Human intestinal tract serves as an alternative infection route for Middle East respiratory syndrome coronavirus. *Sci. Adv.* **3**, eaao4966 (2017).

ScienceAdvances

Human intestinal tract serves as an alternative infection route for Middle East respiratory syndrome coronavirus

Jie Zhou, Cun Li, Guangyu Zhao, Hin Chu, Dong Wang, Helen Hoi-Ning Yan, Vincent Kwok-Man Poon, Lei Wen, Bosco Ho-Yin Wong, Xiaoyu Zhao, Man Chun Chiu, Dong Yang, Yixin Wang, Rex K. H. Au-Yeung, Ivy Hau-Yee Chan, Shihui Sun, Jasper Fuk-Woo Chan, Kelvin Kai-Wang To, Ziad A. Memish, Victor M. Corman, Christian Drosten, Ivan Fan-Ngai Hung, Yusen Zhou, Suet Yi Leung and Kwok-Yung Yuen

Sci Adv **3** (11), eaao4966.
DOI: 10.1126/sciadv.aao4966

感染症研究報告　調査報告書

調査報告書番号	KK-JRC-000016	文献ID	28701419
研究報告の題目	Proviral Features of Human T Cell Leukemia Virus Type 1 in Carriers with Indeterminate Western Blot Analysis Results.		

研究報告の概要	報告企業の意見
○ウェスタンブロット（WB）法の結果が判定保留となったHTLV-1ウイルスキャリアにおけるプロウイルスの特徴 供血者や妊婦を対象としてHTLV-1抗体スクリーニング検査が実施され、抗体が検出された場合は、確認検査としてWB法が実施されている。しかし、WB法では判定保留となることが多い。この原因について、HTLV-1プロウイルスのゲノム配列を解析して検討した。 定量PCR法を用いてHTLV-1プロウイルス量を測定したところ、プロウイルス量の中央値は、WB法で陽性となった検体では0.71コピー/100細胞であったのに対し、WB法の結果が判定保留となった検体（WB判定保留検体）では0.01コピー/100細胞であった。WB判定保留検体における全HTLV-1ゲノムの系統発生解析では、原因の特定には至らなかった。WB判定保留検体から得られたHTLV-1分離株19株のヌクレオチド変異を分析したところ、135塩基の単一ヌクレオチド置換を検出し、置換パターンはG＞A（発生頻度：29%）、C＞T（19%）、T＞C（19%）、A＞G（16%）の4種類であった。最も頻度が高いG＞A置換については、うち64%がGGジヌクレオチドで発生しており、WB判定保留検体における突然変異の原因がAPOBEC3Gにあることが示唆された。さらに、WB判定保留検体から分離した5株において、PolまたはTax（もしくはこの両方）、Env、p12、p30のナンセンス突然変異が認められた。これらの知見は、HTLV-1のプロウイルスが低濃度であること及び突然変異を有することにより抗原産生量が減少し、抗体産生量も低下することで、WB法の結果が判定保留となり得ることを示している。	HTLV-1のプロウイルスが低濃度であること及び突然変異を有することにより抗原産生量が減少し、抗体産生量も低下することで、WB法の結果が判定保留となる可能性が示されたとの報告である。

今後の対応	その他参考事項等
今後も引き続き情報の収集に努める。	

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

VIROLOGY

Proviral Features of Human T Cell Leukemia Virus Type 1 in Carriers with Indeterminate Western Blot Analysis Results

Madoka Kuramitsu,[a] Tsuyoshi Sekizuka,[b] Tadanori Yamochi,[c] Sanaz Firouzi,[c] Tomoo Sato,[d] Kazumi Umeki,[e] Daisuke Sasaki,[f] Hiroo Hasegawa,[f] Ryuji Kubota,[g] Rieko Sobata,[h] Chieko Matsumoto,[h] Noriaki Kaneko,[i] Haruka Momose,[a] Kumiko Araki,[a] Masumichi Saito,[a] Kisato Nosaka,[j] Atae Utsunomiya,[k] Ki-Ryang Koh,[l] Masao Ogata,[m] Kaoru Uchimaru,[c,n] Masako Iwanaga,[o] Yasuko Sagara,[p] Yoshihisa Yamano,[d] Akihiko Okayama,[e] Kiyonori Miura,[q] Masahiro Satake,[h] Shigeru Saito,[r] Kazuo Itabashi,[s] Kazunari Yamaguchi,[a] Makoto Kuroda,[b] Toshiki Watanabe,[t] Kazu Okuma,[a] Isao Hamaguchi[a]

Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan[a]; Pathogen Genomic Center, National Institute of Infectious Diseases, Tokyo, Japan[b]; Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan[c]; Department of Rare Diseases Research, Institute of Medical Science, St. Marianna University School of Medicine, Kawasaki, Japan[d]; Department of Rheumatology, Infectious Diseases and Laboratory Medicine, University of Miyazaki, Miyazaki, Japan[e]; Department of Laboratory Medicine, Nagasaki University Hospital, Nagasaki, Japan[f]; Division of Molecular Pathology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan[g]; Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society, Tokyo, Japan[h]; Department of Special Testing, SRL Inc., Tokyo, Japan[i]; Department of Hematology, Kumamoto University of Medicine, Kumamoto, Japan[j]; Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan[k]; Department of Hematology, Osaka General Hospital of West Japan Railway Company, Osaka, Japan[l]; Department of Medical Oncology and Hematology, Oita University Faculty of Medicine, Oita, Japan[m]; Department of Hematology and Oncology, Research Hospital, Institute of Medical Science, The University of Tokyo, Tokyo, Japan[n]; Department of Frontier Life Science, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan[o]; Japanese Red Cross Kyushu Block Blood Center, Fukuoka, Japan[p]; Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan[q]; Department of Obstetrics and Gynecology, University of Toyama, Toyama, Japan[r]; Department of Pediatrics, Showa University School of Medicine, Tokyo, Japan[s]; Department of Advanced Medical Innovation, Graduate School of Medicine, St. Marianna University, Kawasaki, Japan[t]

ABSTRACT Western blotting (WB) for human T cell leukemia virus type 1 (HTLV-1) is performed to confirm anti-HTLV-1 antibodies detected at the initial screening of blood donors and in pregnant women. However, the frequent occurrence of indeterminate results is a problem with this test. We therefore assessed the cause of indeterminate WB results by analyzing HTLV-1 provirus genomic sequences. A quantitative PCR assay measuring HTLV-1 provirus in WB-indeterminate samples revealed that the median proviral load was approximately 100-fold lower than that of WB-positive samples (0.01 versus 0.71 copy/100 cells). Phylogenic analysis of the complete HTLV-1 genomes of WB-indeterminate samples did not identify any specific phylogenetic groups. When we analyzed the nucleotide changes in 19 HTLV-1 isolates from WB-indeterminate samples, we identified 135 single nucleotide substitutions, composed of four types, G to A (29%), C to T (19%), T to C (19%), and A to G (16%). In the most frequent G-to-A substitution, 64% occurred at GG dinucleotides, indicating that APOBEC3G is responsible for mutagenesis in WB-indeterminate samples. Moreover, interestingly, five WB-indeterminate isolates had nonsense mutations in Pol and/or Tax, Env, p12, and p30. These findings suggest that WB-indeterminate carriers have low production of viral antigens because of a combination of a low

Received 23 April 2017 **Returned for modification** 11 May 2017 **Accepted** 7 July 2017

Accepted manuscript posted online 12 July 2017

Citation Kuramitsu M, Sekizuka T, Yamochi T, Firouzi S, Sato T, Umeki K, Sasaki D, Hasegawa H, Kubota R, Sobata R, Matsumoto C, Kaneko N, Momose H, Araki K, Saito M, Nosaka K, Utsunomiya A, Koh K-R, Ogata M, Uchimaru K, Iwanaga M, Sagara Y, Yamano Y, Okayama A, Miura K, Satake M, Saito S, Itabashi K, Yamaguchi K, Kuroda M, Watanabe T, Okuma K, Hamaguchi I. 2017. Proviral features of human T cell leukemia virus type 1 in carriers with indeterminate Western blot analysis results. J Clin Microbiol 55:2838–2849. https://doi.org/10.1128/JCM.00659-17.

Editor Angela M. Caliendo, Rhode Island Hospital

Address correspondence to Isao Hamaguchi, 130hama@niid.go.jp.

proviral load and mutations in the provirus, which may interfere with host recognition of HTLV-1 antigens.

KEYWORDS nonsense mutation, nucleotide substitution, proviral load, provirus, Western blot indeterminate, human T cell leukemia virus, nucleic acid technology

Human T cell leukemia virus type 1 (HTLV-1) infection is endemic in various regions, including sub-Saharan Africa, the Caribbean, parts of South America, the Middle East, Australo-Melanesia, and the southwestern area of Japan (1, 2). HTLV-1 can be transmitted through prolonged breast feeding, sexual intercourse, and transfusion of contaminated blood. In some African countries, zoonotic transmission to humans by severe bites from simian T cell leukemia virus type 1-infected monkeys has been observed (3). The majority of infected people live without any symptoms; however, in a portion of carriers, HTLV-1 causes adult T cell leukemia (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis, and HTLV-1 uveitis/HTLV-1-associated uveitis after a long period of latency (4).

Diagnosis of HTLV-1 infection is usually made by serological testing at the initial screening of blood donors, in pregnant women, and in suspected HTLV-1-related diseases. A variety of serological screening kits are available, including chemiluminescent enzyme immunoassays (CLEIAs), chemiluminescent immunoassays, and particle agglutination assays, followed by confirmation by Western blotting (WB) (5–9). WB measures the serological reaction to both Gag core proteins (p19, p24, and p53) and the Env protein gp46 (10). In the ProBlot HTLV-1 WB test kit, at least one Gag band and an Env gp46 band should be detected for an HTLV-1-positive result. However, incomplete antibody binding to HTVL-1 Gag or Env is often observed and therefore the result is classified as indeterminate (see Table S1 in the supplemental material).

The proportion of indeterminate WB results is reported to be high in areas such as Zaire (68%) and Central Africa (65.65%) (11, 12). The frequent occurrence of the indeterminate pattern in WB makes it difficult to diagnose the infection correctly. Causes of these indeterminate results have been reported to be cross-reactivity with *Plasmodium falciparum* infection (13–15), infection with HTLV-3 and HTLV-4 (16, 17), and delayed seroconversion with low antibody titers (18–22). In those with WB-indeterminate samples, the indeterminate result is sometimes sustained for a long time (18, 19, 23). Nevertheless, it has been reported that a significant portion of HTLV-1 WB-indeterminate samples are positive for provirus by DNA testing, i.e., 12.5% of WB-indeterminate blood donors in Iran, 9.2% in Brazil, 14.7% (5 of 34) in Argentina, and 42% of patients with neurologic symptoms and 44% of blood donors in the United States (19, 24–27). Thus, in addition to serological testing, proviral DNA detection by quantitative PCR (qPCR) and/or qualitative PCR with HTLV-1-specific primers and a probe against genomic DNA is considered one of the best methods to resolve issues in diagnosis. However, the reason why HTLV-1 provirus-positive blood initially returns an indeterminate result by WB is unclear, especially as it is unlikely that all provirus-positive samples were in the window period of the viral infection prior to antibody formation.

In this study, we assessed the mechanism causing indeterminate WB results by analyzing the complete HTLV-1 genome in WB-indeterminate samples. Furthermore, we evaluated the advantages of HTLV-1 qPCR for cases that were not clearly diagnosed by serological testing.

RESULTS

Provirus detection in WB-indeterminate samples. To detect the HTLV-1 provirus with high sensitivity, we first evaluated the suitable amount of genomic DNA used in HTLV-1 qPCR in eight Japanese laboratories. To assess the detection limit of the qPCR assay in these laboratories, peripheral blood mononuclear cells (PBMCs) that were spiked with low concentrations of HTLV-1-infected cells were analyzed. All laboratories

A

TL-Om1 concentration	Laboratory (ng/reaction)																	
	D (30)	F (50)	A (50)	C (100)	E (100)	B (100)	H (NA)	G (500)	A (500)	F (500)	D (500)	H (1000)	C (1000)	B (1000)	G (1000)	E (2000)		
PC	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
0.05%	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	NT	NT	NT	NT	NT	NT	NT	NT		
0.02%	+ + +	+ + +	+ + +	+ + +	+ + +*	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
0.005%	+* +* +	+* - +	+ - -	+ + +*	+* + +	+* + +	+ + +	+ + +	+ + +	+ + +*	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
0.002%	- +* -	- +* +*	- - -	- +* +*	+* +* +*	+ +* +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
0.0005%	NT	NT	NT	NT	NT	NT	NT	NT	+ + +	- +* -	+* +* +	+ + +	+ +* +	+ + +	+ + +	+ + +		
0.0002%	NT	NT	NT	NT	NT	NT	NT	NT	- - -	- - -	+* +* +*	+ + +	+* - +	+ +* +	+* + +	+ + +*		
PBMC	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -		

B

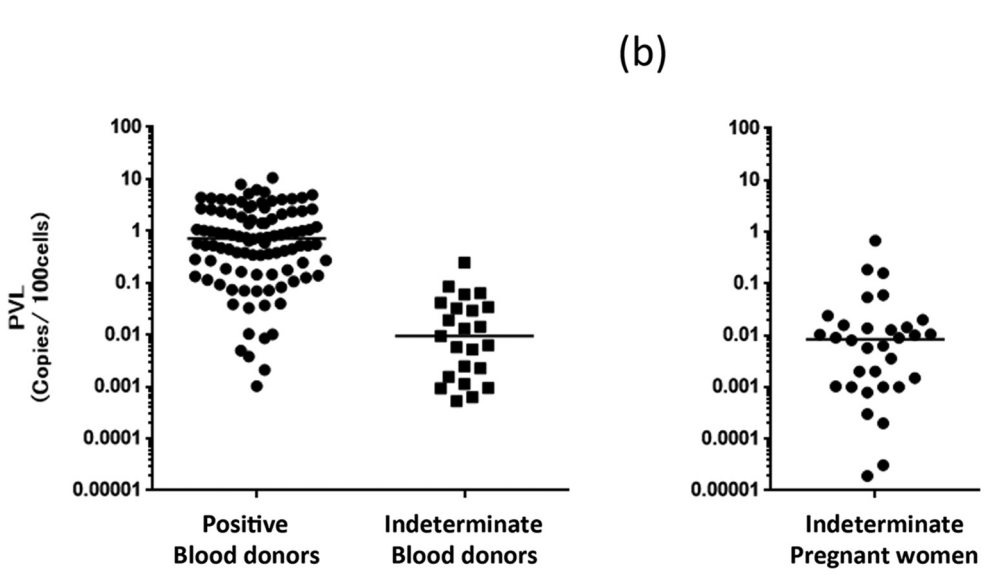


FIG 1 PVLs of WB-indeterminate clinical samples. (A) qPCR was performed three times on different days independently with PBMCs spiked with TL-Om1 cells at concentrations of 0.0002 to 0.05%. Laboratories used different amounts of genomic DNA and their in-house qPCR methods. A number in parentheses under a letter corresponding to a laboratory indicates the amount (in nanograms) of DNA used in the reaction mixture. The PC (positive-control) sample consisted of genomic DNA from 0.8% TL-Om1/PBMC. Tests were performed with duplicate or triplicate wells. A plus sign indicates that all of the wells were positive, a minus sign indicates that all of the wells were negative, and a plus sign with an asterisk indicates that there was at least one negative well in the results. NT, not tested. Gray shading indicates that there was at least one negative result in the test. (B, part a) HTLV-1 PVLs (number of copies per 100 cells) of WB-positive ($n = 100$; left) and WB-indeterminate ($n = 23$; right) blood donors. (B, part b) PVLs of WB-indeterminate pregnant women ($n = 32$). Bars indicate median PVLs.

could detect the provirus at concentrations of approximately 4 copies/10^5 cells when laboratories used >500 ng of genomic DNA in the PCR (Fig. 1A).

We collected genomic DNA from the peripheral blood of pregnant women with indeterminate WB results ($n = 196$) from all over Japan and of blood donors from two geographic areas, one where HTLV-1 infection is endemic ($n = 39$) and the other where it is not ($n = 61$). The frequency of HTLV-1 provirus and the HTLV-1 proviral loads (PVLs) in these WB-indeterminate samples were then measured by the optimized qPCR method (by using 1 μg of genomic DNA). The percentage of provirus-positive blood donors differed according to where the blood was collected (46.2% where HTLV-1 infection is endemic and 8.2% where it is not) (Table 1). Similarly, provirus was detected in 16.5% of WB-indeterminate pregnant women. Among the provirus-positive samples, the median PVL (number of copies per 100 cells) was 0.011 in blood donors ($n = 23$) and 0.008 in pregnant women ($n = 32$) (Fig. 1 and Table 1). Meanwhile, the median PVL of WB-positive blood donors ($n = 100$, a mixture of the two sample areas) was 0.71 copy/100 cells. From these results, the PVL of WB-indeterminate samples was approximately 100-fold lower than that of WB-positive donors (Fig. 1). The antibody titers of provirus-positive WB-indeterminate samples were higher than those of provirus-

TABLE 1 Provirus detection by qPCR and PVLs of WB-indeterminate samples

Sample source and region[a]	Total no. analyzed	No. (%) provirus positive	Median PVL[b] (95% CI[c])
Blood donors			
Endemic	39	18 (46.2)	0.011 (0.002–0.029)
Nonendemic	61	5 (8.2)	
Pregnant women nationwide	194	32 (16.5)	0.008 (0.002–0.014)

[a]Where HTLV-I infection is endemic or nonendemic.
[b]HTLV-1 PVL (number of copies/100 cells).
[c]CI, confidence interval.

negative WB-indeterminate samples ($P < 0.0001$; Fig. S2). A significant correlation between PVLs and antibody titers in the initial screening test of blood donors was not observed (data not shown).

Phylogenetic features of the provirus were not associated with the indeterminate result. In the HTLV-1 screening, we occasionally had samples that the PCR results indicated were positive for HTLV-1; however, these infections could not be confirmed by WB. For example, Matsumoto et al. recently reported that 33 of 600 CLEIA-positive blood donor samples were provirus positive but WB indeterminate and 2 of 600 CLEIA-positive samples were provirus positive but WB negative (28). We hypothesized that genomic features of HTLV-1 may be associated with the indeterminate results of the antibody test.

To investigate the causative phylogenetic feature of HTLV-1 in WB-indeterminate blood donor samples, the full genomic sequences of 114 HTLV-1 WB-positive and 19 WB-indeterminate samples were determined by direct sequencing. A total of 1,085 single nucleotide variants (SNVs) were found in these 133 isolates and four HTLV-1 genomes that were registered as from Japan (ATK-1, ATL-YS, ATL-25, and TL-Om1). A phylogenetic tree was drawn (Fig. 2) with RAxML, which utilizes a maximum-likelihood method. The majority of isolates belonged to the subtype A Japanese (JP) subgroup, while a small portion of isolates belonged to the subtype A transcontinental (TC) subgroup. WB-indeterminate isolates were dispersed throughout both the JP and TC branches (Fig. 2 and Table 2). The frequency of TC-type WB-indeterminate samples in the tree appears relatively high compared with that of the JP type (5 of 10 and 14 of 118, respectively); however, it is difficult to compare the frequencies of those two groups statistically because the geographic background of these pregnant women is unknown. Importantly, distinct WB-indeterminate strains that clustered in specific regions of the phylogenetic tree were not found.

Characteristics of nucleotide substitutions in the HTLV-1 genomes of WB-indeterminate samples. To determine the host enzymes responsible for the mutagenesis of the HTLV-1 genomes in WB-indeterminate samples, such as the APOBEC family, we focused on the nucleotide substitutions in the HTLV-1 genomes. Among the total of 1,085 SNVs found in the HTLV-1 genomes from 114 HTLV-1 WB-positive and 19 WB-indeterminate samples, there were 135 indeterminate WB result-specific single nucleotide substitutions. The most frequent type of substitution was G to A (28.9%), followed by C to T (19.3%), T to C (19.3%), and A to G (16.3%) (Fig. 3A). These four types of substitutions were responsible for 83.8% of the indeterminate WB result-specific substitutions. Moreover, the majority of G-to-A substitutions occurred at GG dinucleotides (64.1%), suggesting that a large portion of these G-to-A substitutions were mediated by APOBEC3G (29) (Fig. 3B).

Characteristics of HTLV-1 genomic sequences in WB-indeterminate samples. We then focused on the mutations associated with viral replication in HTLV-1 from WB-indeterminate samples. To our surprise, among the 19 full HTLV-1 genomic sequences from WB-indeterminate samples, five isolates had nonsense mutations in the coding region of viral proteins, namely, two in Pol, one in Env, one in Tax, two in p12, and one in the p30 sequence (Table 3). Because p30 and p13 use the same coding

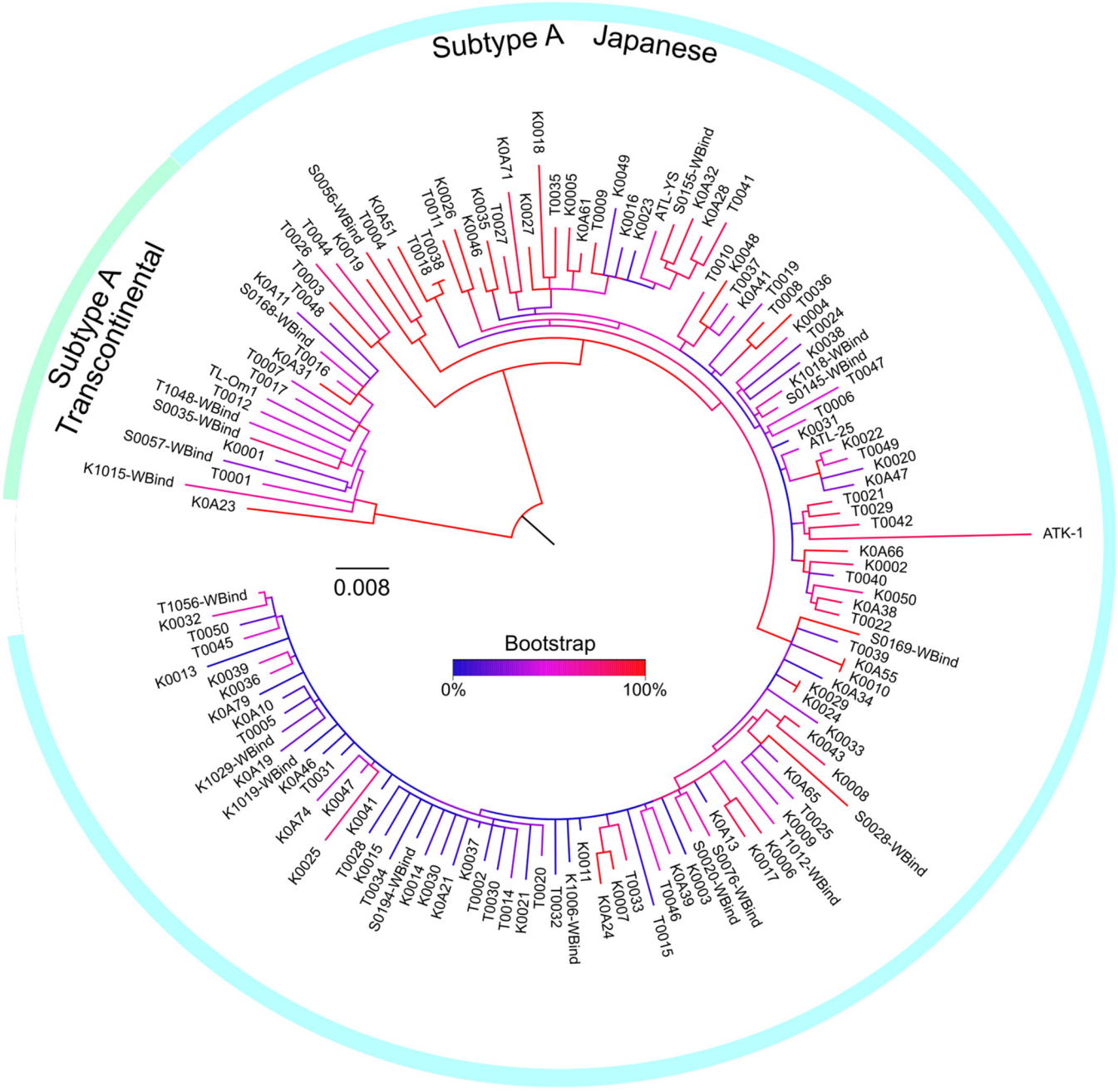


FIG 2 Phylogenetic analysis of HTLV-1 isolates from WB-indeterminate samples. SNVs of 1,085 nucleotide positions in the full HTLV-1 genomes of WB-positive ($n = 114$) and -indeterminate ($n = 19$) samples were analyzed by RAxML, an algorithm that uses the maximum-likelihood method. The tree was drawn by using the GTRGAMMA model. WBind indicates an HTLV-1 sequence from a WB-indeterminate sample. ATK-1 (J02029), ATL-YS (HTU19949), ATL-25 (AB513134), and TL-Om1 (AB979451) are complete HTLV-1 sequences from clinical samples or a cell line derived from Japanese donors.

frame, a p30 W178X mutation (sample K1015) also leads to a p13 W24X mutation. Thus, these HTLV-1 isolates apparently have a fatal replication defect. As shown in Fig. 3C, almost all of the peaks of the electropherograms of nucleotide sequencing of these stop codon mutations were a single peak, suggesting that the HTLV-1 clones with abortive replication constitute the major clone or clones in the carrier. Interestingly, the nonsense mutations in K1015 were mixed with wild-type sequences. In contrast, among the HTLV-1 genomes of 114 WB-positive blood donors, there was only one premature termination in the C-terminal region of Tax (Q334X, accession no. LC209961) and one premature termination in the C-terminal region of p12 (W82X, accession no. LC210033).

TABLE 2 Phylogenetic types of HTLV-1 in WB-positive and -indeterminate samples

Sample source and WB result	Total no. analyzed	No. of HTLV-1 subtype A subgroup:	
		JP	TC
Blood donors			
Positive	114	104	10
Indeterminate	8	6	2
Pregnant women, indeterminate	11	8	3

There were no other premature terminations in the WB-positive samples. This indicated that these genomic mutations with premature termination are possibly a specific feature of WB-indeterminate samples. In addition, all WB-indeterminate samples had many unique mutations in a variety of proteins (Table 4). Including the nonsense mutations in Table 3, on average, there were 4.7 amino acid changes in the provirus of WB-indeterminate samples. Among the unique mutations, there were mutations that cause amino acid charge changes, including Asp, Glu, Arg, and Lys mutations (36%, 30 of 83), and cause structure changes such as a Pro mutation (14%, 12 of 83). These accumulated mutations suggest that there are dramatic changes in the function of viral proteins that may lead to decreased HTLV-1 replication efficiency.

DISCUSSION

We successfully established the HTLV-1 qPCR assay for analysis of the PVLs of WB-indeterminate samples after initially estimating the detection limit of the HTLV-1 qPCR assay. An important feature of HTLV-1 discovered in WB-indeterminate samples was that the PVL of WB-indeterminate samples was generally extremely low. The PVL

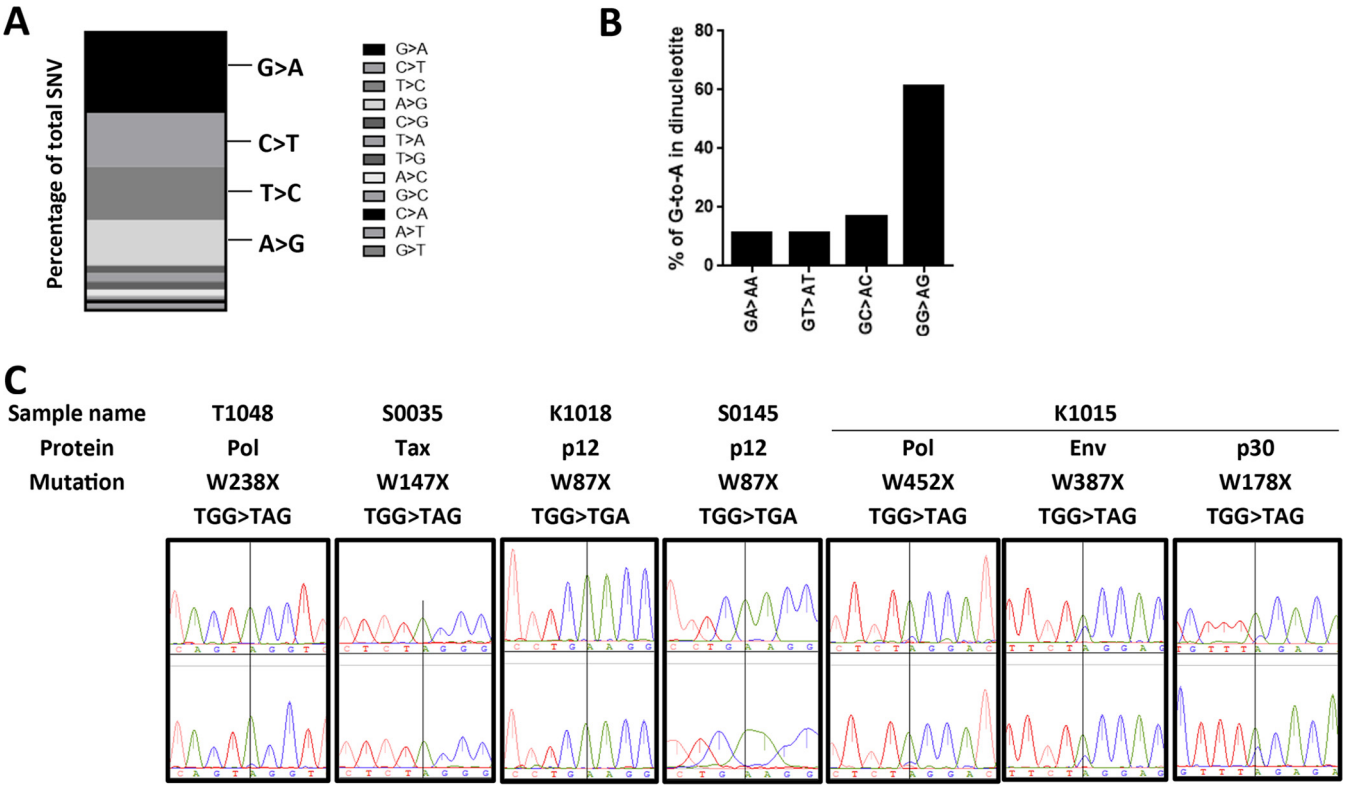


FIG 3 Characteristics of nucleotide substitutions in WB-indeterminate samples. (A) The occurrence of types of nucleotide substitutions is represented as a percentage of the total number of mutations. The four major substitutions, G-to-A, C-to-T, T-to-C, and A-to-G mutations, are indicated. (B) Percentages of second-nucleotide use at G-to-A mutation sites. (C) Electropherogram of stop codon substitutions. Two representative electropherograms per sample are shown. The nucleotide with a vertical line through it is the position of the G-to-A substitution.

TABLE 3 Abortive genetic changes in HTLV-1 genes in WB-indeterminate samples

Sample source and name	PVL[a]	Amino acid mutation(s) in viral protein: Gag	Pro	Pol	Env	Rex	Tax	p12	p30 (p13)	HBZ	Phenotype
Blood donors											
T1048	0.013			W238X							Premature termination
K1015	0.244			W452X	W387X				W178X (W24X)		Premature termination
K1018	0.060							W87X			Premature termination
Pregnant women											
S0035	0.006						W147X				Premature termination
S0145	0.677							W87X			Premature termination

[a]HTLV-I PVL (number of copies per 100 cells).

was approximately 100-fold lower than that of carriers with WB-positive results. Interestingly, there was a geographic difference between the provirus DNA positivity rates of WB-indeterminate blood donors in areas where HTLV-1 infection is not endemic and areas where it is, 8.2 and 46.2%, respectively (Table 1). These rates were as high as those previously reported in other areas of the world, including Iran and the United States. The geographic changes may be produced by the balance of the population of indeterminate samples from the true negative (HTLV-1-uninfected) group, which were originally identified as background in the WB test, and indeterminate samples from the true positive (HTLV-1-infected) group, which were not able to be identified as positive by the WB test. Therefore, the PCR positivity rate will rise in areas where HTLV-1 infection is endemic because the higher number of indeterminate WB results from true positive is increased.

Using more than 100 complete HTLV-1 genome sequences from areas where HTLV-1 infection is endemic and where it is not, we produced an overview of the phylogenic tree of Japanese HTLV-1. Importantly, by adding the HTLV-1 sequences of WB-indeterminate samples to the tree, we revealed that there were no specific subgroups of strains that frequently generate indeterminate WB results in Japan. In other words, one of the causes of indeterminate WB results may be associated with individual HTLV-1 nucleotide mutations rather than the strain of HTLV-1. Although our results are applicable to WB-indeterminate samples from Japanese carriers, the cause of indeterminate WB results in other HTLV-1 strains around the world will be elucidated by precise genomic analysis in further studies. These results may also be useful for the improvement of HTLV-1 diagnostic kits.

It has been reported that HTLV-related viruses or malaria infection cause indeterminate WB results; however, these causes are applicable only in limited areas of the world and Japan is not an area where these pathogens are endemic (13, 15–17). We assessed the cause of indeterminate HTLV-1 WB results by analysis of the entire genomic sequence of HTLV-1 and found that a portion of HTLV-1 strains with indeterminate results have a premature termination codon in viral proteins. These mutations apparently decreased the virus's replication efficiency because the viral proteins could not function like those of the wild-type virus, which possibly led to decreased antigen expression in the long term. We think the mechanism of emergence of these mutated proviruses is that in WB-indeterminate carriers, wild-type virus-infected cells have been eliminated by the host immune system and eventually only mutated viruses with low antigen production remain. This hypothesis is supported by our finding that there remained a faint wild-type sequence in some electropherograms with nonsense mutations (Fig. 3C). Abortive HIV-1 infection was reported previously in samples with indeterminate WB results (30). In addition, a report showed Tax point mutations in HTLV-1 WB-indeterminate samples (31). In our study, premature stop codons were observed not only in Tax but also in various HTLV-1 coding regions, such as Pol, Env, p12, and p30, in WB-indeterminate samples, indicating an association of abortive HTLV-1 strains with indeterminate WB results. In addition, unique mutations were observed not only in the target proteins of WB tests such as Gag and Env but also other

TABLE 4 Genetic changes with unknown significance in HTLV-1 genes in 19 WB-indeterminate samples

Sample source and name	PVL[b]	Amino acid mutation(s) in viral protein:									Nucleotide mutation(s) in LTR	Phenotype
		Gag	Pro	Pol	Env	Rex	Tax	p12	p30	HBZ		
Blood donors												
T1012	0.019		E169D	K93R, T557A	G460R	S134F						Unknown
T1048[a]	0.013	P100S						F54L			490T>G, 559C>T	
T1056	0.032			M701V			H347TfsX18				53_54insA, 116_117insC	Tax frameshift
K1006	0.034	T345N	V16A			G52E, T62M			G191R, R201C			Unknown
K1015[a]	0.244	A9T		V293I, S355C, G482R	V247A, G446R	V29A	G21R, G137R, G259R		S168P		544C>T	
K1018[a]	0.060		Q46E	H503R	T267del				G90E	P63S		
K1019	0.084			G850S	A264V	R7K, S70N			A209T			Unknown
K1029	0.009	A156V, V161I									479delT, 289C>T	Unknown
Pregnant women												
S0020	0.160		Q206R					L64P	F67L	K87R	239T>C	Unknown
S0028	0.006		R166M	I204V	G96D, E179D		G65S, N185Y		R222Q		355G>A	Unknown
S0035[a]	0.006	S113P, P409S	C183Y, G188R	R12Q, G183R		P8S, G166R					319G>A	
S0056	0.014				L267R	T4A		F62L			73A>T, 146C>T	Unknown
S0057	0.016			R259K, I433M		G144D, C162Y		P23S	L26F	R127K	618A>G	Unknown
S0076	0.008			P547S			H347TfsX18		F118S	K35E	53_54insA	Tax frameshift
S0145[a]	0.677			E173K					G90E	P63S	487T>C	
S0155	0.024	S162F		Y678C					T151A		353G>A	Unknown
S0168	0.055			Q812R					G29S		585T>A, 744A>G	Unknown
S0169	0.014		L17F	K855R	L164R							Unknown
S0194	0.059	P103_S104insPP			S81P						587T>C	Unknown

[a]Sample also has abortive mutations.
[b]HTLV-1 PVL (number of copies/100 cells).

TABLE 5 Summary of proviral features of WB-indeterminate samples in this study

No. of samples[a] analyzed	qPCR result	HTLV-1 genome	Type of mutation
239	Provirus negative		
36	Provirus positive	Not determined	
14		Determined	Unique
5			Nonsense

[a]Total of 294.

HTLV-1 proteins. Interestingly, the Tax G137R mutation was observed in K1015. This amino acid is critical for the Tax function of NF-κB signaling (32). Furthermore, Rex T4A (S0056) and P8S (S0035) mutations are located in the RxRE association domain and the Rex T62M (K1006) mutation is located in the Rex multimerization domain (33). This leads us to hypothesize that the cause of indeterminate results is not only the inadequate sensitivity of the diagnostic kit for Env and Gag antibody detection but also the nature of HTLV-1 in WB-indeterminate samples. Mutated HIV-1 can revert back to the wild type after transmission (34, 35). However, we believe that this is not the case in HTLV-1 of WB-indeterminate samples. Generally, after settlement of HTLV-1 infection, HTLV-1 prefers to disseminate through mitotic division of infected cells with cellular DNA polymerase (36). Moreover, PVLs are low in WB-indeterminate samples and replication-incompetent mutations are dominant in a portion of HTLV-1 of WB-indeterminate samples. Thus, HTLV-1 in WB-indeterminate samples almost lost the opportunity to introduce mutations back into the wild type by HTLV-1 reverse transcriptase at transmission.

To summarize our results, WB-indeterminate samples could be divided into four groups on the basis of their PVLs and genomic features (Table 5). The first group is negative for HTLV-1 provirus. This includes true-negative samples and those undetectable by qPCR. In the second group, HTLV-1 provirus is detected despite the extremely low PVL. This possibly includes wild-type HTLV-1 provirus. In the third group, the abortive HTLV-1 strain is dominant. In the fourth group, unique amino acid or nucleotide mutations are present in the provirus. A common property of the provirus of the latter three groups of provirus-positive samples would be an extremely low level of HTLV-1 antigen production. Thus, we could conclude that WB-indeterminate samples tend to have an extremely low level of HTLV-1 antigen expression because of specific features of the HTLV-1 genome. This low antigen level leads to an insufficient antibody titer for the determination of infection by WB. Sustained indeterminate WB results over a prolonged period could be partially explained by this hypothesis (18–20, 23).

Interestingly, APOBEC3G, a host mediator of GG-to-AG substitutions, facilitates the abortion of viral, including HIV-1, replication (37). However, in our study, its function possibly facilitates the survival of HTLV-1 provirus through the decreased production of viral antigens, leading to escape from the host immune system. We think this fits well with the explanation for the reports of the frequent PCR positivity of WB-indeterminate samples (19, 24, 26). Fan et al. analyzed mutations in ATL and reported that among the mutations in ATL, G-to-A is the most frequent and a GG-to-AG substitution was also prominent in all G-to-A mutations (29). They additionally showed the frequent occurrence of stop codon substitutions in the HTLV-1 genome in ATL. Our findings on the mutation status of WB-indeterminate samples are thus in accordance with those reported for ATL. The reason why the same phenomena were observed in the HTLV-1 genome in both ATL and WB-indeterminate samples is unknown. It will be further elucidated through the precise analysis of strategies used by HTLV-1 to continue to reside in carriers.

Finally, our finding that the provirus exists with reduced replication activity in a portion of WB-indeterminate carriers through genetic mutation in the provirus strongly emphasizes the importance of nucleotide amplification testing, such as qPCR, for the diagnosis of HTLV-1 infection.

MATERIALS AND METHODS

Cells and clinical samples. TL-Om1 cells were a kind gift from Kazuo Sugamura (Miyagi Cancer Center Research Institute) (38). TL-Om1 cells were maintained in RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 2 mmol/liter L-glutamine, and 10 ng/ml interleukin-2 (PeproTech, London, United Kingdom). PBMCs were purchased from AllCells Inc. (Alameda, CA, USA). Cryopreserved PBMCs were resuspended in RPMI 1640 supplemented with 10% FBS at 37°C in accordance with the protocol provided by AllCells Inc.

PBMCs from HTLV-1 WB-indeterminate pregnant women were obtained with informed consent. Blood clots from HTLV-1 WB-indeterminate blood donors were obtained in two different areas of HTLV-1 epidemiology, the Kanto Block Blood Center, in an area where HTLV-1 infection is not endemic, including the prefectures of Tokyo and Chiba, and the Kyushu Block Blood Center, in an area where HTLV-1 infection is not endemic, including the prefectures of Kyushu Island. The kit used for initial blood donor screening was Lumipulse Presto HTLV-I (Fujirebio, Tokyo, Japan), one of the CLEIAs. The WB kit used for confirmation of the first screening results was ProBlot HTLV-I (Fujirebio, Tokyo, Japan) (7). Briefly, in ProBlot HTLV-I, bands of p19, p24, and p53 for Gag and gp46 for Env are used for interpretation of the result. The bands were defined by three grades, namely, −, ±, and +. If all bands are −, the result is judged as negative. When Env gp46 and Gag p19, p24, or p53 are +, the result is judged as positive. Band patterns that are neither negative nor positive are judged as indeterminate (Table S1). The antibody titers and profiles of WB-indeterminate patterns of blood donors are listed in Table S2. Information about the kinds of kits used for initial screening of pregnant women was unavailable. In addition, the antibody titers and WB band patterns of pregnant women were unavailable. This study was approved by the ethical review boards of the National Institute of Infectious Diseases (Institutional Review Board approval no. 392).

Eight Japanese laboratories (one national institute [the National Institute of Infectious Diseases], five universities [The University of Tokyo, the St. Marianna University School of Medicine, Nagasaki University, the University of Miyazaki, and Kagoshima University], one Japanese Red Cross laboratory [the Central Blood Institute], and one diagnostic test company [SRL Inc.]) participated in this study.

Preparation of HTLV-1 cell dilutions. Previously, we found that the HTLV-1 copy number of the TL-Om1 genome was 1.8/cell by fluorescence *in situ* hybridization analysis (39). The method used to stain TL-Om1 cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) was previously described (40). CFSE-stained TL-Om1 cells that were resuspended in Cellbanker (TaKaRa Bio, Osaka, Japan) were serially diluted with PBMCs at the concentrations described in Fig. 1 and frozen at −80°C. The concentrations of TL-Om1 cells were analyzed by flow cytometry with a JSAN flow cytometer (Bay Bioscience, Kobe, Japan) (Tables S3 and S4). A series of the same frozen samples packed in dry ice were then provided to the participating laboratories by the National Institute of Infectious Diseases.

Estimation of detection limit of HTLV-1 qPCR. The DNA extraction methods of the laboratories have been described previously (40). The protocols for HTLV-1 qPCR performed in the eight laboratories have also been reported previously (41–47) (Table S5).

HTLV-1 qPCR was performed with purified DNA in laboratories independently three times on different days. To evaluate all of the preparation steps, each measurement began with the extraction of genomic DNA from aliquots of frozen cell samples provided to each laboratory and testing was performed once with the extracted DNA.

Analysis of HTLV-1 genomic sequences. The full-length genomic sequence of HTLV-1 was amplified from four regions by long PCR with the KOD-FX neo polymerase kit (Toyobo, Tokyo, Japan) in accordance with the manufacturer's protocol. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The sequencing PCR was performed with the BigDye ver 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) with sequencing primers in accordance with the manufacturer's protocol. All of the primer sequences used in the long PCR and the sequencing PCR are described in Fig. S1 and Tables S6 and S7. The sequences of PCR products were read from both strands. For sequencing of the GC-rich region of the HTLV-1 genome, equivalent to nucleotides 2099 to 2124 of the ATK-1 (accession no. J02029) reference strain, the dGTP BigDye Terminator v3.0 kit (Applied Biosystems) was used with primers 34F (GGAGATATGTTGCGGGCTTGT) and 41R (GGGAGGTGAGCTTAAA GTGATCTT), respectively, in accordance with the kit protocol. The sequence was determined with an Applied Biosystems 3730 DNA analyzer. Contigs were composed by the sequence assembling software ATGC (GENETYX, Tokyo, Japan). Complete long terminal repeat (LTR) sequences were determined by combining consensus regions of 5′ and 3′ LTR reads as described previously (48). HTLV-1 sequences of T0018 and T0038 were obtained from the same donor on different donation dates.

Phylogenetic analysis. In addition to blood donor samples in which the PVLs were quantitated, a further 23 genomic sequences of HTLV-1 from WB-positive blood donors from the Kyushu Block Blood Center were added to the phylogenetic analysis. For phylogenetic analysis, SNVs were extracted and analyzed with RAxML by the maximum-likelihood method with 1,000 bootstrap samples. The phylogenetic tree was inferred by using the GTRGAMMA model.

Accession number(s). The HTLV-1 nucleotide sequences of WB-indeterminate samples have been submitted to the DNA Data Bank of Japan (DDBJ) and assigned NCBI accession numbers LC185235 to LC185242 and LC192254 to LC192264. The HTLV-1 sequences of WB-positive samples have also been submitted to the DDBJ and assigned NCBI accession numbers LC209958 to LC210071.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00659-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Isao Naruse, Setsuko Sato, Takuo Mizukami, Shuji Izumo, and Shimeru Kamihira for their expert assistance.

This work was supported by the Ministry of Health, Labour and Welfare (MHLW) (H23-sinkou-ippan-016) and the Research Program on Emerging and Re-emerging Infectious Diseases of the Japan Agency for Medical Research and Development, AMED (H26-sinkoujitsuyouka-ippan-013).

We declare no conflicts of interest.

REFERENCES

1. Satake M, Yamaguchi K, Tadokoro K. 2012. Current prevalence of HTLV-1 in Japan as determined by screening of blood donors. J Med Virol 84:327–335. https://doi.org/10.1002/jmv.23181.
2. Gessain A, Cassar O. 2012. Epidemiological aspects and world distribution of HTLV-1 infection. Front Microbiol 3:388. https://doi.org/10.3389/fmicb.2012.00388.
3. Filippone C, Betsem E, Tortevoye P, Cassar O, Bassot S, Froment A, Fontanet A, Gessain A. 2015. A severe bite from a nonhuman primate is a major risk factor for HTLV-1 infection in hunters from Central Africa. Clin Infect Dis 60:1667–1676. https://doi.org/10.1093/cid/civ145.
4. Watanabe T. 2011. Current status of HTLV-1 infection. Int J Hematol 94:430–434. https://doi.org/10.1007/s12185-011-0934-4.
5. Qiu X, Hodges S, Lukaszewska T, Hino S, Arai H, Yamaguchi J, Swanson P, Schochetman G, Devare SG. 2008. Evaluation of a new, fully automated immunoassay for detection of HTLV-I and HTLV-II antibodies. J Med Virol 80:484–493. https://doi.org/10.1002/jmv.21083.
6. Cassar O, Gessain A. 2017. Serological and molecular methods to study epidemiological aspects Of Human T-cell lymphotropic virus type 1 infection. Methods Mol Biol 1582:3–24. https://doi.org/10.1007/978-1-4939-6872-5_1.
7. Miyakoshi H, Sugimoto M, Igarashi H, Honda H, Fujino R, Mizukoshi M. 1992. Improvement of simultaneous detection of antibodies to Gag and envelope antigens of human T-lymphotropic virus type I by Western immunoblot assay. J Clin Microbiol 30:2555–2559. http://jcm.asm.org/content/30/10/2555.long.
8. Nishizono I, Iida S, Suzuki N, Kawada H, Murakami H, Ashihara Y, Okada M. 1991. Rapid and sensitive chemiluminescent enzyme immunoassay for measuring tumor markers. Clin Chem 37:1639–1644.
9. Ikeda M, Fujino R, Matsui T, Yoshida T, Komoda H, Imai J. 1984. A new agglutination test for serum antibodies to adult T-cell leukemia virus. Gan 75:845–848.
10. Anonymous. 1991. AIDS: proposed WHO criteria for interpreting Western blot assays for HIV-1, HIV-2, and HTLV-I/HTLV-II. Bull World Health Organ 69:127–129, 131–133.
11. Filippone C, Bassot S, Betsem E, Tortevoye P, Guillotte M, Mercereau-Puijalon O, Plancoulaine S, Calattini S, Gessain A. 2012. A new and frequent human T-cell leukemia virus indeterminate Western blot pattern: epidemiological determinants and PCR results in Central African inhabitants. J Clin Microbiol 50:1663–1672. https://doi.org/10.1128/JCM.06540-11.
12. Garin B, Gosselin S, de The G, Gessain A. 1994. HTLV-I/II infection in a high viral endemic area of Zaire, Central Africa: comparative evaluation of serology, PCR, and significance of indeterminate Western blot pattern. J Med Virol 44:104–109. https://doi.org/10.1002/jmv.1890440119.
13. Porter KR, Liang L, Long GW, Bangs MJ, Anthony R, Andersen EM, Hayes CG. 1994. Evidence for anti-Plasmodium falciparum antibodies that cross-react with human T-lymphotropic virus type I proteins in a population in Irian Jaya, Indonesia. Clin Diagn Lab Immunol 1:11–15. http://cvi.asm.org/content/1/1/11.long.
14. Mahieux R, Horal P, Mauclere P, Mercereau-Puijalon O, Guillotte M, Meertens L, Murphy E, Gessain A. 2000. Human T-cell lymphotropic virus type 1 gag indeterminate Western blot patterns in Central Africa: relationship to *Plasmodium falciparum* infection. J Clin Microbiol 38:4049–4057. http://jcm.asm.org/content/38/11/4049.long.
15. Hayes CG, Burans JP, Oberst RB. 1991. Antibodies to human T lymphotropic virus type I in a population from the Philippines: evidence for cross-reactivity with Plasmodium falciparum. J Infect Dis 163:257–262. https://doi.org/10.1093/infdis/163.2.257.
16. Calattini S, Betsem E, Bassot S, Chevalier SA, Mahieux R, Froment A, Gessain A. 2009. New strain of human T lymphotropic virus (HTLV) type 3 in a Pygmy from Cameroon with peculiar HTLV serologic results. J Infect Dis 199:561–564. https://doi.org/10.1086/596206.
17. Calattini S, Chevalier SA, Duprez R, Bassot S, Froment A, Mahieux R, Gessain A. 2005. Discovery of a new human T-cell lymphotropic virus (HTLV-3) in Central Africa. Retrovirology 2:30. https://doi.org/10.1186/1742-4690-2-30.
18. Martins ML, Santos AC, Namen-Lopes MS, Barbosa-Stancioli EF, Utsch DG, Carneiro-Proietti AB. 2010. Long-term serological follow-up of blood donors with an HTLV-indeterminate Western blot: antibody profile of seroconverters and individuals with false reactions. J Med Virol 82:1746–1753. https://doi.org/10.1002/jmv.21881.
19. Yao K, Hisada M, Maloney E, Yamano Y, Hanchard B, Wilks R, Rios M, Jacobson S. 2006. Human T lymphotropic virus types I and II Western blot seroindeterminate status and its association with exposure to prototype HTLV-I. J Infect Dis 193:427–437. https://doi.org/10.1086/499273.
20. Okayama A, Stuver S, Iga M, Okamoto M, Mueller N, Matsuoka M, Yamaguchi K, Tachibana N, Tsubouchi H. 2001. Sequential change of virus markers in seroconverters with community-acquired infection of human T lymphotropic virus type I. J Infect Dis 183:1031–1037. https://doi.org/10.1086/319282.
21. Césaire R, Bera O, Maier H, Lezin A, Martial J, Ouka M, Kerob-Bauchet B, Ould Amar AK, Vernant JC. 1999. Seroindeterminate patterns and seroconversions to human T-lymphotropic virus type I positivity in blood donors from Martinique, French West Indies. Transfusion 39:1145–1149. https://doi.org/10.1046/j.1537-2995.1999.39101145.x.
22. Manns A, Murphy EL, Wilks R, Haynes G, Figueroa JP, Hanchard B, Barnett M, Drummond J, Waters D, Cerney M. 1991. Detection of early human T-cell lymphotropic virus type I antibody patterns during seroconversion among transfusion recipients. Blood 77:896–905.
23. Rouet F, Meertens L, Courouble G, Herrmann-Storck C, Pabingui R, Chancerel B, Abid A, Strobel M, Mauclere P, Gessain A. 2001. Serological, epidemiological, and molecular differences between human T-cell lymphotropic virus type 1 (HTLV-1)-seropositive healthy carriers and persons with HTLV-I Gag indeterminate Western blot patterns from the Caribbean. J Clin Microbiol 39:1247–1253. https://doi.org/10.1128/JCM.39.4.1247-1253.2001.
24. Zanjani DS, Shahabi M, Talaei N, Afzalaghaee M, Tehranian F, Bazargani R. 2011. Molecular analysis of human T cell lymphotropic virus type 1 and 2 (HTLV-1/2) seroindeterminate blood donors from northeast Iran: evidence of proviral tax, env, and gag sequences. AIDS Res Hum Retroviruses 27:131–135. https://doi.org/10.1089/aid.2010.0017.
25. Berini CA, Eirin ME, Pando MA, Biglione MM. 2007. Human T-cell lym-

photropic virus types I and II (HTLV-I and -II) infection among seroindeterminate cases in Argentina. J Med Virol 79:69–73. https://doi.org/10.1002/jmv.20731.
26. Costa JM, Segurado AC. 2009. Molecular evidence of human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) infections in HTLV seroindeterminate individuals from Sao Paulo, Brazil. J Clin Virol 44:185–189. https://doi.org/10.1016/j.jcv.2008.12.015.
27. Mangano AM, Remesar M, del Pozo A, Sen L. 2004. Human T lymphotropic virus types I and II proviral sequences in Argentinian blood donors with indeterminate Western blot patterns. J Med Virol 74:323–327. https://doi.org/10.1002/jmv.20172.
28. Matsumoto C, Sagara Y, Sobata R, Inoue Y, Morita M, Uchida S, Kiyokawa H, Satake M, Tadokoro K. 2017. Analysis of HTLV-1 proviral load (PVL) and antibody detected with various kinds of tests in Japanese blood donors to understand the relationship between PVL and antibody level and to gain insights toward better antibody testing. J Med Virol 89:1469–1476. https://doi.org/10.1002/jmv.24802.
29. Fan J, Ma G, Nosaka K, Tanabe J, Satou Y, Koito A, Wain-Hobson S, Vartanian JP, Matsuoka M. 2010. APOBEC3G generates nonsense mutations in human T-cell leukemia virus type 1 proviral genomes *in vivo*. J Virol 84:7278–7287. https://doi.org/10.1128/JVI.02239-09.
30. Georgoulias VA, Malliaraki NE, Theodoropoulou M, Spanakis E, Fountouli P, Tsatsaki D, Kotsaki S, Karvela-Aggelaki A, Malliaraki-Pinetidou E. 1997. Indeterminate human immunodeficiency virus type 1 Western blot may indicate an abortive infection in some low-risk blood donors. Transfusion 37:65–72. https://doi.org/10.1046/j.1537-2995.1997.37197176953.x.
31. Cánepa C, Salido J, Ruggieri M, Fraile S, Pataccini G, Berini C, Biglione M. 2015. Low proviral load is associated with indeterminate Western blot patterns in human T-cell lymphotropic virus type 1 infected individuals: could punctual mutations be related? Viruses 7:5643–5658. https://doi.org/10.3390/v7112897.
32. Smith MR, Greene WC. 1990. Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes. Genes Dev 4:1875–1885. https://doi.org/10.1101/gad.4.11.1875.
33. Younis I, Green PL. 2005. The human T-cell leukemia virus Rex protein. Front Biosci 10:431–445. https://doi.org/10.2741/1539.
34. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, Tang Y, Holmes EC, Allen T, Prado JG, Altfeld M, Brander C, Dixon C, Ramduth D, Jeena P, Thomas SA, St John A, Roach TA, Kupfer B, Luzzi G, Edwards A, Taylor G, Lyall H, Tudor-Williams G, Novelli V, Martinez-Picado J, Kiepiela P, Walker BD, Goulder PJ. 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat Med 10:282–289. https://doi.org/10.1038/nm992.
35. Friedrich TC, Dodds EJ, Yant LJ, Vojnov L, Rudersdorf R, Cullen C, Evans DT, Desrosiers RC, Mothe BR, Sidney J, Sette A, Kunstman K, Wolinsky S, Piatak M, Lifson J, Hughes AL, Wilson N, O'Connor DH, Watkins DI. 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat Med 10:275–281. https://doi.org/10.1038/nm998.
36. Carpentier A, Barez PY, Hamaidia M, Gazon H, de Brogniez A, Perike S, Gillet N, Willems L. 2015. Modes of human T cell leukemia virus type 1 transmission, replication and persistence. Viruses 7:3603–3624. https://doi.org/10.3390/v7072793.
37. Yu Q, Konig R, Pillai S, Chiles K, Kearney M, Palmer S, Richman D, Coffin JM, Landau NR. 2004. Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. Nat Struct Mol Biol 11:435–442. https://doi.org/10.1038/nsmb758.
38. Sugamura K, Fujii M, Kannagi M, Sakitani M, Takeuchi M, Hinuma Y. 1984. Cell surface phenotypes and expression of viral antigens of various human cell lines carrying human T-cell leukemia virus. Int J Cancer 34:221–228. https://doi.org/10.1002/ijc.2910340213.
39. Kuramitsu M, Okuma K, Yamagishi M, Yamochi T, Firouzi S, Momose H, Mizukami T, Takizawa K, Araki K, Sugamura K, Yamaguchi K, Watanabe T, Hamaguchi I. 2015. Identification of TL-Om1, an adult T-cell leukemia (ATL) cell line, as reference material for quantitative PCR for human T-lymphotropic virus 1. J Clin Microbiol 53:587–596. https://doi.org/10.1128/JCM.02254-14.
40. Kuramitsu M, Okuma K, Yamochi T, Sato T, Sasaki D, Hasegawa H, Umeki K, Kubota R, Sobata R, Matsumoto C, Kaneko N, Naruse I, Yamagishi M, Nakashima M, Momose H, Araki K, Mizukami T, Mizusawa S, Okada Y, Ochiai M, Utsunomiya A, Koh KR, Ogata M, Nosaka K, Uchimaru K, Iwanaga M, Sagara Y, Yamano Y, Satake M, Okayama A, Mochizuki M, Izumo S, Saito S, Itabashi K, Kamihira S, Yamaguchi K, Watanabe T, Hamaguchi I. 2015. Standardization of quantitative PCR for human T-cell leukemia virus type 1 in Japan: a collaborative study. J Clin Microbiol 53:3485–3491. https://doi.org/10.1128/JCM.01628-15.
41. Sobata R, Matsumoto C, Uchida S, Suzuki Y, Satake M, Tadokoro K. 2015. Estimation of the infectious viral load required for transfusion-transmitted human T-lymphotropic virus type 1 infection (TT-HTLV-1) and of the effectiveness of leukocyte reduction in preventing TT-HTLV-1. Vox Sang 109:122–128. https://doi.org/10.1111/vox.12263.
42. Ueno S, Umeki K, Takajo I, Nagatomo Y, Kusumoto N, Umekita K, Morishita K, Okayama A. 2012. Proviral loads of human T-lymphotropic virus type 1 in asymptomatic carriers with different infection routes. Int J Cancer 130:2318–2326. https://doi.org/10.1002/ijc.26289.
43. Miyazato P, Yasunaga J, Taniguchi Y, Koyanagi Y, Mitsuya H, Matsuoka M. 2006. De novo human T-cell leukemia virus type 1 infection of human lymphocytes in NOD-SCID, common gamma-chain knockout mice. J Virol 80:10683–10691. https://doi.org/10.1128/JVI.01009-06.
44. Watanabe M, Ohsugi T, Shoda M, Ishida T, Aizawa S, Maruyama-Nagai M, Utsunomiya A, Koga S, Yamada Y, Kamihira S, Okayama A, Kikuchi H, Uozumi K, Yamaguchi K, Higashihara M, Umezawa K, Watanabe T, Horie R. 2005. Dual targeting of transformed and untransformed HTLV-1-infected T cells by DHMEQ, a potent and selective inhibitor of NF-kappaB, as a strategy for chemoprevention and therapy of adult T-cell leukemia. Blood 106:2462–2471. https://doi.org/10.1182/blood-2004-09-3646.
45. Takenouchi N, Yamano Y, Usuku K, Osame M, Izumo S. 2003. Usefulness of proviral load measurement for monitoring of disease activity in individual patients with human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis. J Neurovirol 9:29–35. https://doi.org/10.1080/13550280390173418.
46. Kamihira S, Dateki N, Sugahara K, Hayashi T, Harasawa H, Minami S, Hirakata Y, Yamada Y. 2003. Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count. Clin Lab Haematol 25:111–117. https://doi.org/10.1046/j.1365-2257.2003.00503.x.
47. Nagai M, Yamano Y, Brennan MB, Mora CA, Jacobson S. 2001. Increased HTLV-I proviral load and preferential expansion of HTLV-I Tax-specific $CD8^+$ T cells in cerebrospinal fluid from patients with HAM/TSP. Ann Neurol 50:807–812. https://doi.org/10.1002/ana.10065.
48. Cassar O, Einsiedel L, Afonso PV, Gessain A. 2013. Human T-cell lymphotropic virus type 1 subtype C molecular variants among indigenous Australians: new insights into the molecular epidemiology of HTLV-1 in Australo-Melanesia. PLoS Negl Trop Dis 7:e2418. https://doi.org/10.1371/journal.pntd.0002418.

感染症研究報告　調査報告書

調査報告書番号	KK-JRC-000017	文献ID	29019306
研究報告の題目	Detection of a chikungunya outbreak in Central Italy, August to September 2017		

研究報告の概要

〇中央イタリアにおけるチクングニヤアウトブレイク（2017年8月～9月）
ラツィオ州ローマ県の沿岸部にあるアンツィオで土着性チクングニヤ熱のアウトブレイクが発生した。これはイタリアにおいて2度目のアウトブレイクとなる。
2017年9月6日、7日、イタリアの国立リファレンスラボラトリー・アルボウイルス感染症研究室は、38.0℃以上の熱、重度の関節痛、発疹を呈する患者3名の血清及び尿検体を入手した。当該患者は休暇でアンツィオに滞在していた際に発症した。患者は同じ宿舎に滞在し、いずれも発症前の2週間にチクングニヤ熱、デング熱及びジカ熱の流行地域への訪問歴はなかった。発症はそれぞれ8月5日、11日及び9月2日であった。3名ともチクングニヤウイルス感染が確認された。
地域保健局は同集団感染の事例を受け、疫学的サーベイランスを強化した。2017年9月20日現在、地域のサーベイランスシステムに報告された土着性チクングニヤ熱の確定症例は86例となっている。これらの患者は、アンツィオの住人または発症前の15日間にアンツィオへの訪問歴を有する者であった。
最初の確定例3名が滞在していたアンツィオの住宅付近で採取したヒトスジシマカのプール検体が、PCR法によりチクングニヤウイルス陽性であることが判明した。患者の1名から検出されたウイルスのエンベロープ（E）1領域の塩基配列は、カから検出されたウイルス株（LT908476）と100%一致した。また、パキスタンで流行している、A226Vが未変異の東・中央・南アフリカ株（ECSA）と完全に一致したことも示された。系統発生解析では、当該患者らの塩基配列は、世界の様々な地域から得られたECSA株の配列とクラスターを形成した。
9月8日に、イタリアは進行中のアウトブレイクについてEU諸国に警告するため、Early Warning Response Systemを通じて第一回目の警報を発表した。対象の自治体では、ベクターコントロール活動を行い、住民に対しカの刺咬予防や、繁殖場所の排除手段を講じることについて勧告した。さらに、供血者の臨床評価、ラツィオ州に住む供血者の供血後情報の収集及び2017年8月1日以降にアンツィオを訪れた者について28日間の供血延期措置を講じた。

報告企業の意見

イタリアにおける2度目のチクングニヤ熱のアウトブレイクがローマ県の沿岸地域であるアンツィオで発生し、対象地域では現在、ベクターコントロール、サーベイランスの強化及び供血制限を行っているとの報告である。

今後の対応

日本赤十字社では、輸血感染症対策として献血時に海外渡航歴の有無を確認し、帰国（入国）後4週間は献血不適としている。2006年に国内でチクングニヤ熱の輸入症例が確認されたため、渡航歴確認の徹底を図っている。また、チクングニヤ熱の既往歴がある場合、治癒後6ヵ月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。

その他参考事項等

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

Detection of a chikungunya outbreak in Central Italy, August to September 2017

Giulietta Venturi[1,2], Marco Di Luca[1,2], Claudia Fortuna[1], Maria Elena Remoli[1], Flavia Riccardo[1], Francesco Severini[1], Luciano Toma[1], Martina Del Manso[1], Eleonora Benedetti[1], Maria Grazia Caporali[1], Antonello Amendola[1], Cristiano Fiorentini[1], Claudio De Liberato[3], Roberto Giammattei[4], Roberto Romi[1], Patrizio Pezzotti[1], Giovanni Rezza[1], Caterina Rizzo[1]

1. Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy
2. These authors contributed equally to this article and share first authorship
3. Istituto Zooprofilattico Sperimentale Lazio e Toscana, Rome, Italy
4. Local Health Authority Roma 6, Albano, Italy

Correspondence: Caterina Rizzo (caterina.rizzo@iss.it)

Citation style for this article:
Venturi Giulietta, Di Luca Marco, Fortuna Claudia, Remoli Maria Elena, Riccardo Flavia, Severini Francesco, Toma Luciano, Del Manso Martina, Benedetti Eleonora, Caporali Maria Grazia, Amendola Antonello, Fiorentini Cristiano, De Liberato Claudio, Giammattei Roberto, Romi Roberto, Pezzotti Patrizio, Rezza Giovanni, Rizzo Caterina. Detection of a chikungunya outbreak in Central Italy, August to September 2017. Euro Surveill. 2017;22(39):pii=17-00646. https://doi.org/10.2807/1560-7917.ES.2017.22.39.17-00646

Article submitted on 19 eptember 2017 / accepted on 28 September 2017 / published on 28 September 2017

An autochthonous chikungunya outbreak is ongoing near Anzio, a coastal town in the province of Rome. The virus isolated from one patient and mosquitoes lacks the A226V mutation and belongs to an East Central South African strain. As of 20 September, 86 cases are laboratory-confirmed. The outbreak proximity to the capital, its late summer occurrence, and diagnostic delays, are favouring transmission. Vector control, enhanced surveillance and restricted blood donations are being implemented in affected areas.

Outbreak identification and investigation

On 6 and 7 September 2017, the National reference Laboratory for arboviral infections based at the National Institute of Health, Italy, received serum and urine samples from three patients with a history of high fever (›38°C), severe joint pain and an itching skin rash. Symptoms had started while they were on holiday near the coastal town of Anzio, in the province of Rome, Lazio region (ca 58 km from Rome). The dates of symptom onset were 5 and 11 August and 2 September respectively. The patients lived in the same home, and none had travelled to chikungunya, dengue or Zika endemic areas in the two weeks before symptom onset. The two patients who developed symptoms at the beginning of August were chikungunya IgM positive and the infection was confirmed through a neutralisation test (PRNT). The third patient, who was symptomatic at the time of sample collection, was IgM positive and PRNT borderline (PRNT50 ≥ 1:10). Chikungunya virus was detected by RT-PCR followed by nested PCR in both serum and urine and was isolated from urine.

The regional health authorities immediately implemented measures around this initial cluster, which are described in the national plan for chikungunya surveillance and control [1]. The three patients had referred to other individuals in their neighbourhood who had developed similar symptoms since the beginning of August. Epidemiological surveillance was enhanced to identify additional cases (case finding trough general practitioners (GPs), paediatricians, emergency rooms and hospitals). As of 20 September 2017, 86 confirmed autochthonous cases of chikungunya were reported to the regional surveillance system; the investigation is ongoing. These were found among people living in Anzio, or people who had travelled there 15 days before symptom onset. Cases from Rome were also identified, with either travel history to Anzio or to other endemic areas [2]. The primary viraemic case who imported the virus into the region has not been identified so far.

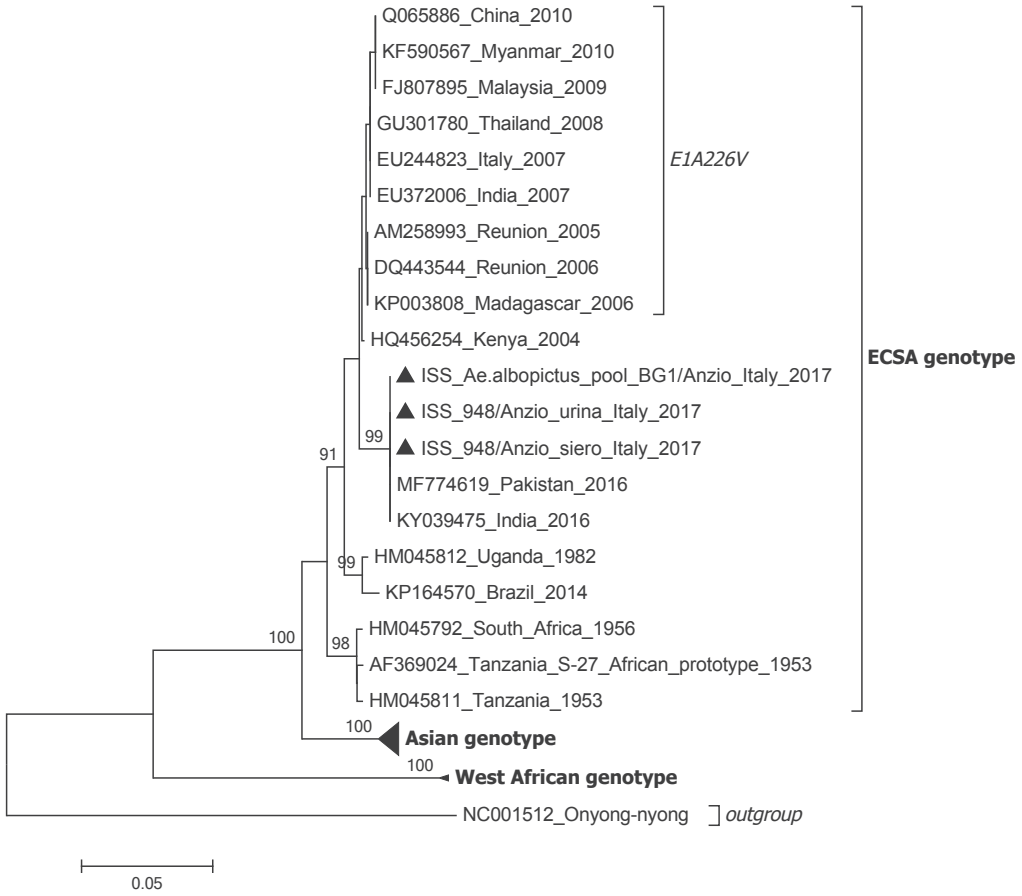
Entomological investigation

On 7 and 8 September 2017, adult mosquitoes were collected in the Anzio municipality by using BG Sentinel traps baited with BG-Lure (Biogents, Regensburg, Germany) and captured directly using handheld electric aspirators on vegetation or on exposed skin. Larval collections were performed using dippers or droppers by inspecting artificial containers (removable or non-removable), as potential larval breeding sites. Adults and larvae of *Ae. albopictus* (tiger mosquito) and *Culex pipiens* were found in the Anzio municipality area. Mosquito adults were sorted into 12 pools to determine the species, distinguish males from females and to test for chikungunya virus. The number of specimens in each pool ranged from 1 to 13. Among 53 mosquitoes, one pool of 12 *Ae. albopictus* females which had been collected near the house of the first three cases identified in Anzio was found to be chikungunya virus positive by PCR. The virus was isolated.

Phylogenetic analysis of patient and mosquito sequences

Figure

Neighbour-joining phylogenetic analysis of sequences derived from chikungunya virus positive samples obtained in this study, Italy, August–September 2017



ESCA: East Central South African.

The phylogenetic tree was constructed using Tamura-Nei model with 1,000 bootstrap reiterations. For each sequence in the tree, GenBank accession number/country of origin of the infection/year of the infection are reported. Sequences characterised in this study are indicated by a black triangle (for the patient samples 'siero' indicates a sequence derived from a serum sample (GenBank number: LT908478), and 'urina', a sequence from a urine sample (LT908477); the GenBank number for the sequence from mosquitoes is LT908476). Sequence amplicons were obtained using method reported in Edwards C. et al., J Clin Virol. 2007 [11]. The scale bar indicates the percentage of diversity. Bootstrap values over 90% obtained from 1,000 replicate trees are shown for key nodes.

Two clusters are highlighted in the Figure. The first, identifiable as 'ESCA genotype', indicates sequences of the ESCA genotype. The second cluster, identifiable as 'E1 A226V', includes all sequences with the A226V mutation in the envelope (E)1 gene, a mutation, which is involved in the increased susceptibility of Aedes albopictus for infection and transmission of chikungunya virus.

Sequences of the PCR amplicons of the virus envelope (E)1 gene from the patient (GenBank numbers: LT908477 and LT908478) and from the mosquitoes were identical (GenBank number: LT908476), and also showed a 100% similarity with the sequence of a chikungunya East Central South African (ECSA) strain involved in an ongoing epidemic in Pakistan [3,4], which does not carry the A226V mutation. In a phylogenetic analysis, the Italian sequences also clustered with ESCA strain sequences obtained from different parts of the world (Figure).

Control measures

On 8 September Italy launched a first alert through the Early Warning Response System (EWRS) in order to alert other European Union countries about the ongoing outbreak. In the affected municipality, vector control activities against larvae and adult mosquitoes were performed and the population was advised to take measures to avoid mosquito bites and to remove all potential breeding sites from their gardens. Moreover, measures such as clinical evaluation of blood donors, gathering post-donation information for donors residing in the Lazio region, and application of 28 days deferral of donors who visited the municipality of Anzio since 1 August 2017 were reinforced [5].

Background

Chikungunya is a self-limiting mosquito-borne viral disease characterised by arthralgia/arthritis primarily

of the wrists, knees, ankles and small joints of the extremities lasting from days to months. Between one third and half of the patients develop a generally non-pruritic maculopapular rash primarily of the trunk and limbs 1–12 days after the onset of arthritis that resolves in 7–10 days. Myalgia, fatigue, fever and lymphadenopathy are common [6]. Complications are rare; however, Chikungunya leads to (self-perceived) long-term sequelae in a considerable proportion of patients [7]. The incubation period ranges from 1 to 10 days (average 3 days) [8].

The aetiological agent is a single strand, positive sense RNA virus of the Alphavirus genus, Togaviridae family of which three main genotypes have been identified: Asian, West African and ECSA [8]. The virus is transmitted by mosquitoes of the *Aedes* spp. primarily *Ae. aegypti* and *Ae. albopictus*. Transmission through transfusion/transplantation has been demonstrated in animal models.

Chikungunya is endemic in Africa, south-east Asia, the Indian subcontinent, the Pacific region and in tropical regions of the Americas (since 2013). In Europe, autochthonous cases, which were linked to imported cases, were detected in France in the Var department, in September 2010 and in the city of Montpellier, Hérault department, in October 2014. In August 2017, a cluster of locally acquired cases consisting of four confirmed and one probable case was also described in the Var department [8].

Discussion

The outbreak described here is the second autochthonous chikungunya outbreak detected in Italy. The first outbreak, with more than 200 reported cases, occurred between July and September 2007 in the north east of the country, near the Adriatic coast, in the province of Ravenna [9]. Subsequent to it, Italy set up a national plan for the surveillance and control of the disease [1]. The aim of this surveillance system is to monitor imported cases and local transmission, identify outbreaks in a timely fashion, and to prevent transmission from substances of human origin (SoHO). The surveillance is enhanced during the high vector activity season (1 June–31 October). The competent vector, *Ae. albopictus*, which was first detected in north-western Italy in 1990 [9], is now widely established throughout the country, including the Lazio region. Overall between 2014 and 2016, a total of 128 possible/probable/confirmed imported cases of chikungunya have been notified to the Italian health authorities (mean: 43 cases/year; range: 25 cases in 2015 and 70 cases in 2014). Most cases had a travel history to Central and South America [1].

Anzio, one of the locations where autochonous chikungunya cases were currently detected, is a holiday resort less than one hour drive away from Rome. Many families visit the area until the beginning of the school year, which in 2017 was on 14 September. During the summer, the population density increases in the area owing to the easy commute from Rome. Viraemic individuals exposed in August 2017 are likely to have travelled around resulting in new introductions in receptive areas and challenging the implementation of control measures. The delayed identification of the autochthonous cases in Anzio, a coastal recreational area close to the capital towards the end of summer may have triggered the local transmission in Rome. This suggests an extension of the outbreak and the presence of secondary multifocal transmission chains [10].

The lack of access to closed summer houses after mid-September may hamper vector control activities. Summer 2017 has been characterised by particularly high temperatures and by an unusual dry period from May to August, factors that would normally prevent high mosquito density. However, other concomitant factors favoured the activity and abundance of the tiger mosquito including the presence of numerous peridomestic 'man-made' breeding sites within constantly irrigated private gardens.

The identification of the viral strain involved in the current Italian outbreak as one very close to the ECSA strain involved in the ongoing epidemic in Pakistan is not surprising, even if this virus it is not carrying the A226V mutation, which has been involved in the increased susceptibility of *Ae. albopictus* for infection and transmission of chikungunya virus. Other yet undefined mutations might be responsible for the vector competence of *Ae. albopictus*, allowing it to sustain the present outbreak. The fact that we found chikungunya RNA in a small size mosquito pool, suggests a high level of virus circulation in the area. The investigation is still ongoing and, due to enhanced surveillance and testing, further cases have been identified, with as of 22 September, 102 laboratory-confirmed cases.

Acknowledgements

The authors would like to thank Dr Francesco Paolo Maraglino and Dr Patrizia Parodi from the Italian Ministry of Health and all health professionals from local and regional health authorities for collaborating.

Conflict of interest

None declared.

Authors' contributions

CR, GV, MDL, coordinate the national integrated Plan for Chikungunya surveillance and control, interpreted the data and wrote the manuscript. MDM, and MGC manage the web-based platform for data collection and analysed the data. GV, MER, EB, CF, AA and CF performed laboratory investigation. CDL, MDL, RR, FS and LT performed the entomological surveillance and monitoring. RG, CR, FR, and PP performed the epidemiological investigation. GR interpreted the data and critically revised the manuscript. All authors reviewed and approved the final manuscript.

References

1. Ministry of Health. Italian National plan for the surveillance and control of Chikungunya, Dengue and Zika 2017. [Accessed 18 Sep 2017]. Available from: http://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero&id=3013
2. Lazio Region. [Accessed 25 Sep 2017]. Available from https://www.regione.lazio.it/rl_main/?vw=newsDettaglio&id=4112latest
3. Mallhi TH, Khan YH, Khan AH, Tanveer N, Qadir MI. First chikungunya outbreak in Pakistan: a trail of viral attacks. New Microbes New Infect. 2017;19:13-4. https://doi.org/10.1016/j.nmni.2017.05.008 PMID: 28663798
4. ProMED-mail. Chikungunya (31): Asia (Pakistan). Archive Number 20170916.5320238. 16 Sep 2017. Available from: http://www.promedmail.org/
5. National Blood Center. Chikungunya fever: blood donations suspended in Rome. [Accessed 18 Sep 2017]. Available from: http://www.centronazionalesangue.it/notizie/chikungunya-chikv
6. Heyman DL. Control of Communicable Diseases Manual. 19th Ed. Washington, DC: American Public Health Association; 2008.
7. van Aalst M, Nelen CM, Goorhuis A, Stijnis C, Grobusch MP. Long-term sequelae of chikungunya virus disease: A systematic review. Travel Med Infect Dis. 2017;15:8-22. https://doi.org/10.1016/j.tmaid.2017.01.004 PMID: 28163198
8. European Centre for Disease Prevention and Control (ECDC). Cluster of autochtonous chikungunya cases in France – 23 August 2017. Stockholm: ECDC; 2017.
9. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, Panning M, et al. CHIKV study group. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet. 2007;370(9602):1840-6. https://doi.org/10.1016/S0140-6736(07)61779-6 PMID: 18061059
10. European Centre for Disease Prevention and Control (ECDC). Clusters of autochthonous chikungunya cases in Italy, 14 September 2017. Stockholm: ECDC; 2017.
11. Edwards CJ, Welch SR, Chamberlain J, Hewson R, Tolley H, Cane PA, et al. Molecular diagnosis and analysis of Chikungunya virus. J Clin Virol. 2007;39(4):271-5. https://doi.org/10.1016/j.jcv.2007.05.008 PMID: 17627877

License and copyright

感染症研究報告　調査報告書

調査報告書番号	KK-kaketsuken-20180112	文献ID	28905395
研究報告の題目	St. Louis encephalitis virus possibly transmitted through blood transfusion -Arizona, 2015		

研究報告の概要

セントルイス脳炎ウイルスが輸血により感染した初めての報告。
セントルイス脳炎はほとんどが不顕性感染で、1%以下の確率で脳炎、髄膜炎、急性弛緩性麻痺といった神経性疾患を発症する。
これまで輸血感染の報告はなく、スクリーニングも実施されていなかった。

・69歳男性、2015年に腎臓移植を受けるために入院。メチルプレドニゾロン、バシリキシマブ投与。臓器移植後、免疫抑制剤投与。
・患者は移植後1、2、20、21日目の計4回それぞれ異なるドナーから赤血球輸血を受けた。34日目に退院。35日目にセントウイルス脳炎を発症し再度入院。105日目に退院したが、記憶喪失、健忘症などの後遺症あり(その後回復)。
・35日目(発症日)と43日目の血清でセントウイルス脳炎中和抗体が有意に上昇(20倍⇒1280倍)
・患者は入院していたので外出はほとんどなし。移植前の発熱、蚊への曝露、州外への旅行もなし。
・腎臓ドナーについて、フラビウイルス感染の形跡なし(直前の血清でRNAも抗体も検出されていない。リンパ節にも抗原なし)。同一ドナーから他の臓器(心臓、肝臓、右腎)を移植された3人の患者も感染していなかった。
・赤血球輸血は4名のドナーから4回であった。輸血した赤血球は残っていなかったが、移植後20日目の輸血ドナーが、後日(77日後)採血によりセントルイス脳炎抗体陽性であることが確認された。ドナーは不顕性感染であった。このドナーの血漿は他にも77歳女性に投与されていた。この女性は投与2週後にホスピスに移り、3日後に死亡した。セントルイス脳炎を疑う所見はなかった。
・患者の自宅周辺、病院周辺の蚊からはセントルイス脳炎ウイルスは検出されなかったが、抗体陽性となった20日目の輸血ドナー自宅周辺の蚊からウイルス陽性の蚊が見つかった。

報告企業の意見

セントルイス脳炎ウイルスは、フラビウイルス科フラビウイルス属に属し、フラビウイルスはプラス鎖一本鎖RNAをゲノムとするエンベロープウイルスであり、ウイルス粒子は直径40～60nmである。セントルイス脳炎は米国、カナダ、メキシコ、中南米に広く分布し、セントルイス脳炎ウイルスの伝播には、蚊から鳥、鳥から蚊という感染サイクルを示し、家禽や野鳥およびイエカ種の蚊が感染サイクルに含まれている。ヒトや家畜は感染した蚊から刺咬されることで感染すると考えられる。セントルイス脳炎ウイルスと同じフラビウイルス科フラビウイルス属に属するウエストナイルウイルスでは輸血後感染の報告齢はあるが、これまでセントルイス脳炎ウイルスによる輸血後の感染例の報告はなかった。今回、米国において赤血球輸血後の感染例が報告された。

今後の対応

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。

その他参考事項等

(注意)
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

St. Louis encephalitis virus possibly transmitted through blood transfusion—Arizona, 2015

Heather Venkat,[1,2,3] Laura Adams,[2,4] Rebecca Sunenshine,[3,4] Elisabeth Krow-Lucal,[1,5] Craig Levy,[3] Tammy Kafenbaum,[3] Tammy Sylvester,[3] Kirk Smith,[6] John Townsend,[6] Melissa Dosmann,[7] Hany Kamel,[8] Roberto Patron,[7] Matthew Kuehnert,[9] Pallavi Annambhotla,[9] Sridhar V. Basavaraju,[9] Ingrid B. Rabe,[5] and the SLEV Transmission Investigation Team

BACKGROUND: St. Louis encephalitis virus is a mosquito-borne flavivirus that infrequently causes epidemic central nervous system infections. In the United States, blood donors are not screened for St. Louis encephalitis virus infection, and transmission through blood transfusion has not been reported. During September 2015, St. Louis encephalitis virus infection was confirmed in an Arizona kidney transplant recipient. An investigation was initiated to determine the infection source.

STUDY DESIGN AND METHODS: The patient was interviewed, and medical records were reviewed. To determine the likelihood of mosquito-borne infection, mosquito surveillance data collected at patient and blood donor residences in timeframes consistent with their possible exposure periods were reviewed. To investigate other routes of exposure, organ and blood donor and recipient specimens were obtained and tested for evidence of St. Louis encephalitis virus infection.

RESULTS: The patient presented with symptoms of central nervous system infection. Recent St. Louis encephalitis virus infection was serologically confirmed. The organ donor and three other organ recipients showed no laboratory or clinical evidence of St. Louis encephalitis virus infection. Among four donors of blood products received by the patient via transfusion, one donor had a serologically confirmed, recent St. Louis encephalitis virus infection. Exposure to an infected mosquito was unlikely based on the patient's minimal outdoor exposure. In addition, no St. Louis encephalitis virus-infected mosquito pools were identified around the patient's residence.

CONCLUSION: This investigation provides evidence of the first reported possible case of St. Louis encephalitis virus transmission through blood product transfusion. Health care providers and public health professionals should maintain heightened awareness for St. Louis encephalitis virus transmission through blood transfusion in settings where outbreaks are identified.

St. Louis encephalitis virus (SLEV) is a mosquito-borne flavivirus closely related to West Nile virus (WNV). These viruses share the same mosquito vectors, and their associated disease presentations are clinically indistinguishable.[1] Like WNV, SLEV is primarily maintained and amplified through cycles between

ABBREVIATIONS: CSF = cerebrospinal fluid; MCES = Maricopa County Environmental Services; PRNT = confirmatory plaque reduction neutralization testing; SLEV = St Louis encephalitis virus; WNV = West Nile virus.

From the [1]Epidemic Intelligence Service Program, Division of Scientific Education and Professional Development, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia; the [2]Arizona Department of Health Services; and the [3]Maricopa County Department of Public Health, Phoenix, Arizona; the [4]Office of Public Health Preparedness and Response, Career Epidemiology Field Officer Program, CDC, Atlanta, Georgia; the [5]CDC Division of Vector-Borne Diseases, Fort Collins, Colorado; the [6]Maricopa County Environmental Services Vector Control Division; and the [7]Division of Infectious Diseases, Mayo Clinic, Phoenix, Arizona; the [8]Blood Systems, Inc., Scottsdale, Arizona; and the [9]Division of Healthcare Quality Promotion, CDC, Atlanta, Georgia.

Address reprint requests to: Heather Venkat, 150 North 18th Avenue, Suite 100, Phoenix, AZ 85007; e-mail: hvenkat@cdc.gov.

This investigation was supported by the Centers for Disease Control and Prevention, the Arizona Department of Health Services, the Maricopa County Department of Public Health, and Blood Systems, Inc., as part of their routine activity.

Received for publication May 11, 2017; revision received July 25, 2017; and accepted July 27, 2017.

doi:10.1111/trf.14314

TRANSFUSION 2017;57;2987–2994

Culex species mosquitoes and avian hosts, and their geographic and temporal distribution dictates the occurrence of human infections.[2-5] Most SLEV infections are asymptomatic, but they also can result in a nonspecific febrile illness.[3] Less than 1% of human SLEV infections lead to severe neuroinvasive disease, which can present as encephalitis, meningitis, or acute flaccid paralysis; individuals ages 55 years and older are at higher risk for developing SLEV neuroinvasive disease.[3,6-8] SLEV infection can be diagnosed by molecular or serologic testing. The viremic period is transient; thus, ribonucleic acid (RNA) is seldom detectable in acute infections that are evaluated after the onset of symptoms, and serology is the mainstay of diagnosis. However, because antibodies against SLEV and WNV readily cross-react on immunoglobulin (Ig)M diagnostic tests, confirmatory neutralizing antibody testing is required to identify the specific infecting flavivirus.[9]

SLEV was first identified in St. Louis, Missouri, in 1933, and more than 50 outbreaks have been reported in the United States since then.[10,11] Reports of SLEV neuroinvasive disease declined considerably after WNV was first detected in 1999; however, isolated cases and limited outbreaks of SLEV disease still occur sporadically in the United States.[1,2,12] SLEV is a nationally notifiable infectious disease. In 2015, Arizona state and local health authorities identified an outbreak of SLEV during which infection was confirmed in 23 symptomatic persons. Arizona was the only US state to report human SLEV disease cases to the Centers for Disease Control and Prevention (CDC) that year. In addition, acute WNV disease cases were also confirmed, making this the first documented concurrent outbreak of WNV and SLEV in the United States.[13] The majority of SLEV disease cases were reported in Maricopa County, which is the largest urban area in Arizona. Although the blood supply in the United States has attained an unprecedented level of safety, it remains vulnerable to emerging infectious agents.[14] WNV transmission by blood transfusion in the United States has been well documented and is rarely reported since the implementation of routine screening of blood donors for WNV infection by nucleic-acid testing (NAT).[15-17] Blood donor screening for Zika virus, another related flavivirus, was implemented in 2016.[18] SLEV transmission by blood transfusion has not previously been reported.[17] There are no US Food and Drug Administration (FDA)-licensed blood donor screening tests for SLEV.

During September 2015, the United Network for Organ Sharing alerted CDC of suspected neuroinvasive SLEV disease in a kidney transplant recipient in Maricopa County on the basis of the detection of SLEV IgM antibodies in his serum. This patient is referred to hereinafter as the SLEV recipient. The organ recipient care teams, Arizona Department of Health Services, and Maricopa County Department of Public Health were notified. The objectives of the ensuing public health investigation were to confirm the diagnosis of SLEV infection in the recipient and to determine the source of his infection.

MATERIALS AND METHODS

SLEV recipient

The SLEV recipient was interviewed regarding exposure history and clinical course, and his medical records were reviewed. Residual serum specimens collected before organ transplantation and after neuroinvasive disease symptom onset, as well as residual cerebrospinal fluid (CSF) collected after symptom onset were obtained for SLEV and WNV testing. There were no remaining specimens collected between day of transplant and symptom onset.

Case definition

An SLEV disease case was classified according to clinical and laboratory criteria stipulated in the national case definition for reporting of arboviral diseases.[19] In addition, any persons who had laboratory evidence of recent SLEV infection within 4 weeks after receipt of an organ or blood component from a donor with evidence of recent SLEV infection was considered to have a possible transplant-transmitted or transfusion-transmitted infection, respectively. SLEV infection was considered confirmed if there was molecular detection of SLEV RNA or if serologic testing was positive for SLEV IgM with the detection of neutralizing antibodies to SLEV at a titer 4-fold higher than WNV.

Organ donor and other organ recipients

The organ donor's medical records were reviewed, and residual serum, plasma, and lymph node DNA lysate collected before organ recovery were obtained for flavivirus testing. Other recipients of organs from the same donor were contacted to obtain symptom history and serum specimens for SLEV and WNV testing.

Blood donors and other blood product recipients

Medical records were reviewed to determine whether the SLEV recipient had received any blood products before symptom onset. The blood collection center initiated a lookback investigation to determine whether there were any remaining in-date co-components that needed to be quarantined and tested for SLEV RNA. The donors of all identified blood products were contacted to obtain symptom history, residence address at the time of donation, and serum specimens for SLEV testing.

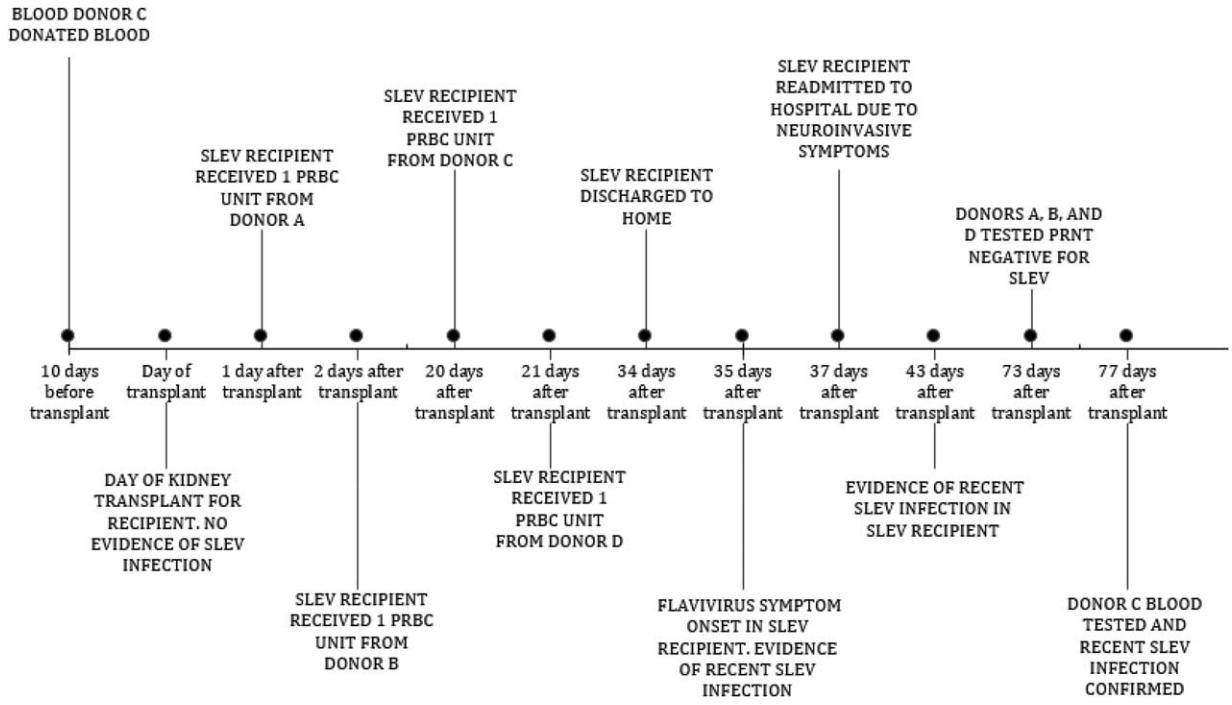


Fig. 1. Investigation timeline of possible St. Louis encephalitis virus transmission through blood transfusion relative to the timing of transplantation of the infected kidney recipient: Arizona, 2015.

Laboratory testing

Serum and CSF samples were tested for the presence of anti-SLEV and anti-WNV IgM and IgG using an enzyme-linked immunosorbent assay at the state public health laboratory, and for anti-WNV IgM using a microsphere-based immunosorbent assay at the CDC.[20] For serum specimens with IgM test results reported as positive or nonspecific, confirmatory plaque reduction neutralization testing (PRNT) using a 90% reduction in the number of plaques (PRNT90) for SLEV and WNV was performed at the CDC.[21] Tissue specimens were tested by real-time polymerase chain reaction (RT-PCR) and immunohistochemistry at the CDC.

Mosquito surveillance

The Maricopa County Environmental Services (MCES) Vector Control Division routinely conducted mosquito surveillance in 2015 for WNV and SLEV because of the circulation of both viruses in the region. Traps were placed throughout Maricopa County to collect *Culex* species mosquitoes. MCES recorded the number and location of mosquito traps and tested all mosquitoes captured using RT-PCR for both WNV and SLEV; mosquitoes from the same trap were tested in pools.[22] To determine whether SLEV was circulating in the areas in which the SLEV patient might have been exposed to mosquitoes, suggesting a greater likelihood of vector-borne transmission, MCES reviewed data from mosquito surveillance conducted during the 30 days before the date of his illness onset for both the area of his residence and the hospital where he was admitted. Similarly, MCES reviewed the data for areas of residence of any implicated donors for 30 days before organ or blood donation. A 5-mile radius around each of the sites was chosen based on the average maximum flight range combined for both adult *C. tarsalis* and *C. quinquefasciatus* mosquitoes from a previous dispersal study.[23]

RESULTS

SLEV recipient

The SLEV recipient was a 69-year-old man who was admitted to the hospital in late July 2015 for a kidney transplant because of end-stage renal disease, which was attributed to diabetic nephropathy and hypertension (Fig. 1). He received methylprednisolone and induction therapy with basiliximab to prevent organ rejection; tacrolimus was added 2 days after transplant. The patient received transfusions of 4 units of leukocyte-reduced red blood cells (pRBCs) on Days 1, 2, 20, and 21 after transplant and was discharged home 34 days after transplant.

The day after discharge, he experienced headache, fever, fatigue, nausea, diarrhea, shortness of breath, and chills, which progressed to rigors. Upon initial consultation, his physicians attributed his symptoms to bacteremia

TABLE 1. West Nile virus and St. Louis encephalitis virus serologic laboratory results for St. Louis encephalitis virus recipient before and after transplant

			SLEV		WNV	
Event	Date	Specimen	MIA	PRNT	MIA	PRNT
Before transplant	Jun 23	Serum	NS	<10	NS	<10
Day of transplant	Jul 28	Serum	NS	<10	NS	<10
Day of symptom onset	Sep 1 (Day 35)*	Serum	Positive	20	Negative	<10
After symptom onset	Sep 9 (Day 43)*	Serum	NS	1,280	NS	<10
	Sep 10 (Day 44)*	Cerebrospinal fluid	Negative	NA	Negative	NA
	Oct 9 (Day 73)*	Serum	Positive	20,480	Negative	<10

NS = nonspecific (i.e., a sample that reacts with the negative antigen such that the result using the viral antigen cannot be interpreted); NA = not done; MIA = microsphere immunoassay.
* After transplant.

secondary to ureteral stent removal; however, he was readmitted 37 days after transplant, when his symptoms progressed to lower extremity paralysis and respiratory distress, requiring a tracheostomy. By Day 43, the patient's mental status began to deteriorate. Serum and CSF specimens were collected on Day 43 to test for possible flavivirus infection, and empiric antibiotics were started for possible bacterial infection. The patient received intravenous immunoglobulin and interferon therapy from Day 44 to Day 48 after transplant and 1 pRBC unit each on Days 49 and 56. On Day 51, the patient exhibited gradual motor function improvement, and he had cognitive improvement by Day 59. The patient was discharged to home on Day 105 and experienced some neurologic sequelae after discharge, including memory loss and weakness.

Residual serum specimens collected 16 days before and on the day of his transplant, respectively, were obtained for testing. The serum specimens had nonspecific results on WNV and SLEV IgM testing but had no detectable neutralizing antibodies to SLEV or WNV, ruling out prior infection. The IgM results likely reflect either prior flavivirus infection or reactivity to other serum factors. Testing of subsequent samples, which were collected on the day of symptom onset and beyond, confirmed seroconversion on PRNT as well as a greater than four-fold increase in SLEV neutralizing antibody titers between serum collected on Day 35 (PRNT = 20) and Day 43 (PRNT = 1280). Neutralizing antibody titers against SLEV were also more than four-fold higher than titers against WNV (Table 1). The SLEV recipient's presentation and test results thus met the clinical and laboratory criteria for neuroinvasive SLEV infection. Upon subsequent interview, the patient reported no febrile illness, exposure to mosquitoes, or travel out of state before transplant. The SLEV recipient spent minimal time outdoors during the weeks before symptom onset, because he was hospitalized with limited mobility. The only noted history of flavivirus exposure was receipt of a yellow fever vaccination in 1965. The patient continued to recover; and, by April 2016, he was reportedly back to neurological baseline.

Organ donor and other organ recipients

The cadaveric donor of the left kidney transplanted into the SLEV recipient died in July 2015 in Illinois. His heart, liver, and right kidney were also recovered and transplanted. According to his medical record, he had no symptoms suggestive of flavivirus infection. The heart and liver recipients were Illinois residents, and the right kidney recipient was an Arizona resident; none of these recipients had symptoms compatible with flavivirus infection after transplantation. Organ donor serum collected 1 day before organ recovery contained no detectable SLEV RNA, and IgM antibody test results were negative. There was no SLEV RNA or SLEV antigen detected in residual lymph node tissue by RT-PCR or immunohistochemistry, respectively. Serum specimens collected 6 weeks after organ transplant from the heart recipient and right kidney recipient had no evidence of recent infection. No specimens were available for testing from the liver recipient.

Blood donors and other blood product recipients

The four pRBC units that the SLEV recipient received before symptom onset were collected from four blood donors (Donors A, B, C, and D) whose blood products were delivered by transfusion on Day 1 (Donor A), Day 2 (Donor B), Day 20 (Donor C), and Day 21 (Donor D) after transplant. The donated blood products had been screened by WNV NAT at the blood collection agency and were negative. There were no remaining blood products available for testing. Serum was collected from these donors from 73 to 85 days after the date of donation for SLEV and WNV testing. Specimens from Donors A, B, and D contained no detectable IgM or neutralizing antibodies against SLEV or WNV. Serum from Donor C, collected 77 days after donation, tested positive for SLEV and negative for WNV IgM by microsphere-based immunosorbent assay. The specimen from blood Donor C had detectable neutralizing antibodies against SLEV (PRNT = 320) but not against WNV (PRNT < 10). This donor did not report any symptoms compatible with flavivirus infection before or after donating blood.

Fig. 2. Mosquito surveillance* for St. Louis encephalitis virus in a 5-mile radius of case locations and donor location for a 30-day period before recipient symptom onset and donor blood donation: Maricopa County, Arizona, 2015. *Fifty traps were located within each 5-mile radius, and only traps that contained mosquitoes are shown; empty traps are not displayed.

The recipient of the fresh-frozen plasma component from Donor C's donation was a 77-year-old woman who received a transfusion after she was admitted to the hospital in late July 2015. She had altered mental status before the blood transfusion and received multiple units of fresh-frozen plasma and platelets while admitted. After approximately 2 weeks of hospitalization, she was transferred to hospice, where she died 3 days later. Her primary diagnoses upon discharge to hospice were toxic metabolic encephalopathy, subdural hematoma, and possible hemolytic anemia/thrombotic thrombocytopenic purpura. The cause of death was not suspected to be a flavivirus infection. No autopsy was conducted. Although no specimens were available for testing, a medical records review did not indicate febrile illness or exacerbation of neurologic features after the transfusion.

Mosquito surveillance

Approximately 50 traps had been placed within a 5-mile radius of the SLEV recipient's residence, Donor C's residence, and the hospital where the SLEV recipient was admitted. Traps were observed for mosquitoes by MCES on a weekly basis. No SLEV-infected mosquitoes were identified from 11 mosquito pools from traps collected within a 5-mile radius around the index patient's residence (Fig. 2) (only traps that trapped live mosquitoes are shown in the figure). No SLEV-infected mosquitoes were identified from 37 mosquito pools from traps collected within a 5-mile radius around the hospital location. Ten SLEV-infected mosquitoes (nine *C. quinquefasciatus* and one *C. tarsalis* species) were identified from 112 mosquito pools from traps collected within a 5-mile radius around Donor C's residence.

DISCUSSION

To our knowledge, this is the first reported case of possible SLEV transmission through blood product transfusion. The patient experienced a clinical illness compatible with SLEV infection 15 days after receiving blood products from an asymptomatic donor with serologically confirmed, recent SLEV infection. The interval between transfusion and symptom onset is consistent with the estimated incubation period for mosquito-transmitted SLEV infection (range, 5-15 days), which has been documented as

prolonged (median, 13.5 days) in immunocompromised recipients with transfusion-associated WNV infection.[16] The timing of SLEV infection after the implicated transfusion is also supported by seroconversion from neutralizing antibodies being undetectable before the transfusion to being detectable and progressively increasing after the transfusion. This case was likely detected because of enhanced surveillance and testing systems in place for the concurrent WNV and SLEV outbreaks in the region.[13] Mosquito surveillance data demonstrated the detection of SLEV infected mosquitoes around Donor C's residence but not around the SLEV recipient's residence or the hospital, indicating that the SLEV recipient might have been at lower risk of mosquito-borne exposure. However, these data were not directly comparable, because many more mosquito pools were tested in the region of the blood donor's residence. In addition, the SLEV recipient spent all of his potential exposure period indoors while hospitalized, except for 1 day spent at home. Serologic test results indicating recent SLEV infection in both the index patient, after the implicated transfusion, and Blood Donor C support SLEV transmission through Blood Donor C's blood products.

Human SLEV infection is most commonly acquired from *Culex* species mosquito bites; however, other less common routes of transmission are possible. One case of laboratory-acquired SLEV infection has been previously described.[24] Although transmission of SLEV through blood transfusion has not been documented in the published literature, transmission of flavivirus infections through blood transfusion is known to occur.[15,25-27] In Arizona, blood products are routinely screened for WNV by NAT.[18,28] There is no FDA requirement or AABB standard for screening blood products for SLEV, and there are no SLEV NAT assays commercially available and approved for blood donor screening.

The benefit of blood product screening for bloodborne pathogens depends on several factors, including disease incidence and severity of outcome; screening has proven effective in reducing transfusion transmission of WNV, for example.[29] Reported SLEV disease incidence nationwide is extremely low; an average of seven SLEV disease cases were reported annually in the United States during 2004 through 2013 compared with an average of 2540 WNV disease cases reported annually during that same period. This would limit the benefit of screening blood donors for SLEV infection.[30] The low US incidence makes the positive predictive value of any SLEV blood product screening test much lower than that for WNV. The duration of donor SLEV infectious risk is assumed to be similar to that for WNV; and, in this case, the local blood collection agency issued a recommendation once confirmatory testing was completed for the asymptomatic blood donor for a 120-day donor deferral period based on the same period stipulated for WNV NAT-positive blood donors.[31]

This investigation had multiple limitations. There were no residual specimens from Blood Donor C's implicated donation to confirm the presence of SLEV in any of the donated blood products, which would have strengthened evidence for transfusion-related transmission of SLEV. We also could not definitely state when Blood Donor C's SLEV viremia occurred, because we only had serologic confirmation of recent infection. Specimens were also unavailable for the plasma recipient, because the investigation took place several weeks after she had died.

This investigation provides evidence to support the possibility that SLEV, like WNV, can be transmitted through blood products, reinforcing the importance of public health surveillance for SLEV disease, especially during an outbreak. Public health officials could determine whether there is an increased risk for SLEV transmission in an area and alert physicians to consider SLEV if there is neuroinvasive disease in a blood transfusion recipient. This should prompt a public health investigation to determine whether the infection might have been transfusion or transplant derived. Current public health surveillance data may under represent the true burden of SLEV disease because of the infrequent availability of SLEV laboratory testing and cross-reactivity with WNV testing. In the absence of systematic data on the risk of transfusion transmission of SLEV, there is currently no justification for routine blood screening; however, continued vigilance for cases like that described herein should inform risk-based decisions on whether further mitigation measures are required. Health care providers and public health professionals should maintain heightened awareness for SLEV transmission through blood transfusion in settings in which SLEV transmission is identified.

SLEV TRANSMISSION INVESTIGATION TEAM

The St. Louis Encephalitis Virus Transmission Investigation Team: Melissa Kretschmer, Lia Koski, Andrew Strumpf, and the Maricopa County Department of Public Health; Hayley Yaglom, Kenneth Komatsu, Lydia Plante, and the Arizona Department of Health Services; Dan Damien and Maricopa County Environmental Services Vector Control Division; Janna Huskey, Hasan Khamash, and Mayo Clinic Hospital; Michael Harmon and the Gift of Hope Organ and Tissue Donor Network; Debbie Freeman and the Illinois Department of Public Health; Tiana Riley and the University of Chicago Medical Center; Becca Craven and the Kovler Organ Transplantation Center; Toni Slaughter and United Blood Services; and Kristine Bisgard and the Centers for Disease Control and Prevention.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

1. Reimann CA, Hayes EB, DiGuiseppi C, et al. Epidemiology of neuroinvasive arboviral disease in the United States, 1999-2007. Am J Trop Med Hyg 2008;79:974-9.
2. Reeves WC, Hammon WM, Longshore WA Jr, et al. Epidemiology of the arthropod-borne viral encephalitides in Kern County, California, 1943-1952. Publ Public Health Univ Calif 1962;4:1-257.
3. Monath TP, Tsai TF. St. Louis encephalitis: lessons from the last decade. Am J Trop Med Hyg 1987;37:40S-59S.
4. McLean RG, Webb JP, Campos EG, et al. Antibody prevalence of St. Louis encephalitis virus in avian hosts in Los Angeles, California, 1986. J Am Mosq Control Assoc 1988;4:524-8.
5. Day JF. Predicting St. Louis encephalitis virus epidemics: lessons from recent, and not so recent, outbreaks. Annu Rev Entomol 2001;46:111-38.
6. Brinker KR, Paulson G, Monath TP, et al. St Louis encephalitis in Ohio, September 1975: clinical and EEG studies in 16 cases. Arch Intern Med 1979;139:561-6.
7. Lindsey HS, Calisher CH, Mathews JH. Serum dilution neutralization test for California group virus identification and serology. J Clin Microbiol 4:503-10.
8. Centers for Disease Control and Prevention (CDC). St. Louis encephalitis epidemiology & geographic distribution [cited 2017 May 10]. Atlanta (GA): CDC; 2015. Available from: http://www.cdc.gov/sle/technical/epi.html
9. Martin DA, Noga A, Kosoy O, et al. Evaluation of a diagnostic algorithm by using immunoglobulin M enzyme-linked immunosorbent assay to differentiate human West Nile virus and St. Louis encephalitis virus infections during the 2002 West Nile virus epidemic in the United States. Clin Diagn Lab Immunol 2004;11:1130-3.
10. Lumsden LL. St. Louis encephalitis in 1933; observations on epidemiological features. Public Health Rep 1958;73:340-53.
11. Kopp A, Gillespie TR, Hobelsberger D, et al. Provenance and geographic spread of St. Louis encephalitis virus. MBio 2013; 4:e00322-13.
12. Nash D, Mostashari F, Fine A, et al. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med 2001;344:1807-14.
13. Venkat H, Krow-Lucal E, Hennessey M, et al. Concurrent outbreaks of St. Louis encephalitis virus and West Nile virus disease—Arizona, 2015. MMWR Morb Mortal Wkly Rep 2015;64:1349-50.
14. Chamberland ME. Emerging infectious agents: do they pose a risk to the safety of transfused blood and blood products? Clin Infect Dis 2002;34:797-805.
15. Harrington T, Kuehnert MJ, Kamel H, et al. West Nile virus infection transmitted by blood transfusion. Transfusion 2003;43:1018-22.
16. Pealer L, Marfin AA, Petersen LR, et al. Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med 2003;349:1236-45.
17. Stramer SL, Hollinger FB, Katz LM, et al. Emerging infectious disease agents and their potential threat to transfusion safety. Transfusion 2009;49(Suppl 2):1S-29S.
18. US Food and Drug Administration (FDA). Guidance for industry: donor screening recommendations to reduce the risk of transmission of Zika virus by human cells, tissues, and cellular and tissue-based products [cited 2017 May 10]. Silver Spring (MD): FDA; 2016. Available from: https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/tissue/ucm488582.pdf
19. Centers for Disease Control and Prevention (CDC). Arboviral diseases, neuroinvasive and non-neuroinvasive 2015 case definition [cited 2017 May 10]. Atlanta (GA): CDC; 2015. Available from: http://wwwn.cdc.gov/nndss/conditions/arboviral-diseases-neuroinvasive-and-non-neuroinvasive/case-definition/2015/
20. Johnson AJ, Noga AJ, Kosoy O, et al. Duplex microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin M antibodies. Clin Diagn Lab Immunol 2005;12:566-74.
21. Johnson AJ, Cheshier RC, Cosentino G, et al. Validation of a microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin m antibodies. Clin Vaccine Immunol 2007;14:1084-93.
22. Sutherland GL, Nasci RS. Detection of West Nile virus in large pools of mosquitoes. J Am Mosq Control Assoc 2007;23:389-95.
23. Verdonschot PFM, Besse-Lototskaya AA. Flight distance of mosquitoes (Culicidae): a metadata analysis to support the management of barrier zones around rewetted and newly constructed wetlands. Limnologica 2014;45:69-79.
24. Von Magnus H. Laboratory infection with St. Louis encephalitis virus. Acta Pathol Microbiol Immunol Scand 1950;27: 276-82.
25. Lederman E, Warkentien T, Bavaro M, et al. Transfusion-related transmission of yellow fever vaccine virus—California, 2009. MMWR Morb Mortal Wkly Rep 2010;59:34-7.
26. Tambyah PA, Koay E, Poon M, et al.; Transfusion-Transmitted Dengue Infection Study Group. Dengue hemorrhagic fever transmitted by blood transfusion. N Engl J Med 2008; 359:1526-7.
27. Motta IJF, Spencer BR, Cordeiro da Silva SG, et al. Evidence for transmission of Zika virus by platelet transfusion. N Engl J Med 2016;375:1101-3.
28. US Food and Drug Administration (FDA). Guidance for industry: use of nucleic acid tests to reduce the risk of transmission of West Nile virus from donors of whole blood and blood components intended for transfusion [cited 2017 Jun 15]. Silver Spring (MD): FDA; 2009. Available from: http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM189464.pdf

29. Custer B, Busch MP, Marfin AA, et al. The cost-effectiveness of screening the US blood supply for West Nile virus. Ann Intern Med 2005;143:486-92.
30. Centers for Disease Control and Prevention (CDC). West Nile virus final cumulative maps and data for 1999-2015 [cited 2017 Jun 15]. Atlanta (GA): CDC; 2016. Available from: http://www.cdc.gov/westnile/statsmaps/cummapsdata.html
31. US Food and Drug Administration (FDA). Guidance for industry: assessing donor suitability and blood and blood product safety in cases of known or suspected West Nile virus infection [cited 2017 Jun 15]. Silver Spring (MD): FDA; 2005. Available from: http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm080286.pdf

感染症研究報告　調査報告書

調査報告書番号	KK-FRI-E17-01	文献ID	
研究報告の題目	JAPANESE ENCEPHALITIS - CHINA (HONG KONG)		

研究報告の概要

香港の保健当局は、致命的な転帰に至る可能性のある蚊を媒介とする日本脳炎について、輸血を介して罹患した世界初の症例に取り組んでいる。
医師によると、他の2人の患者は症状を示さなかったが、1例は今月初旬（2017年7月）に手術を受けた後、脳出血で死亡した。
世界的にも前例のない症例により、日本脳炎の検査を要求しない現在の献血方針に注目が集まったが、保健当局はシステムの変更を行わなかった。
University of Hong Konの微生物学者であるYuen Kwok-yung教授は、日本脳炎は感染の可能性が非常に低いため、献血のスクリーニングを開始する必要はないと述べた。「これは非常にまれであり、不幸な症例である」と彼は述べた。肺移植手術によって、患者は日本脳炎ウイルスに対して脆弱になる可能性があると付け加えた。「医学的観点からは、香港で日本脳炎ウイルス感染者が急増しない限り、マススクリーニング検査を開発する必要はない」と述べた。
2017年までに発生した症例は3例であり、通常はウイルスに感染してから4～14日間後に、感染した100人のうち1人が頭痛、高熱、頸部硬直などの症状を発現する。それは脳腫脹および死につながる可能性がある。yung教授は、日本脳炎ウイルスのために赤十字社の血液バンクをチェックすることは事実上不可能であり、公衆へ警告は必要ないと付け加えた。「今回の事例が、将来一般の人々が献血することを妨げないことを望む」と述べた。赤十字社のLee Cheuk-kwong博士は、健康に関するアンケートに加えて、ドナーは献血後に起きたあらゆる症状を報告するべきだと提案した。
調査では、ドナーはTin Shui Wai 在住の46歳男性で、Kwun Tongで働いていており、2017年5月29日に赤十字社の移動献血サービスを通じて血液を提供していたことが明らかになった。彼は全ての要求された健康検査に合格し、日本脳炎の症状を示さなかった。ドナーは2017年5月にロンドンを訪問したが、蚊に刺された記録はない。保健センターのWong Ka-hing博士は、彼が現地で感染した可能性は非常に高いと語った。
2017年6月20日、患者は感染した血液を輸血され、出血により手術後の2017年7月4日に死亡した。患者は日本脳炎の検査を受けていないが、ウイルス感染の症状は見られなかった。
クイーン・メアリーで白血病治療を受けている別の患者は、感染した血液を2017年6月2日に輸血されたが、日本脳炎の症状を呈すことなく同年7月14日に退院した。

報告企業の意見

日本脳炎はコガタアカイエカによって媒介され、日本脳炎ウイルスにより発症する4類感染症である。通常、ヒトからヒトへの感染はなく、増幅動物（ブタ）の体内でいったん増えて血液中に出てきたウイルスを、蚊が吸血し、その上でヒトを刺した時に感染する。本報告によれば、日本脳炎ウイルスに感染したドナーから提供された血液を介して感染した、世界初の症例である。患者は肺移植手術中に輸血を受けたが、University of Hong Konの微生物学専門家によると、肺移植手術によって患者が日本脳炎ウイルスに対しより脆弱になった状態であると述べた。さらに、同専門家は「日本脳炎ウイルスに感染した患者が急増しない限り、スクリーニング法を開発する必要はない」と述べている。ドナーが提供した血液に対し日本脳炎ウイルスの検査は実施してこなかったが、保険当局による体制の変更は行われなかった。
本報告は、重大な感染症かつ新規感染経路に該当すると考えられ、感染症定期報告の対象と判断する。

今後の対応

ヒト血液を原料とする血漿分画製剤とは直接関連がないことから、現時点で当該生物由来製品に関し、措置等を行う必要はないと判断する。

その他参考事項等

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

Japanese Encephalitis Virus Transmitted Via Blood Transfusion, Hong Kong, China

Vincent C.C. Cheng,[1] Siddharth Sridhar,[1] Shuk-Ching Wong, Sally C.Y. Wong, Jasper F.W. Chan, Cyril C.Y. Yip, Chi-Hung Chau, Timmy W.K. Au, Yu-Yan Hwang, Carol S.W. Yau, Janice Y.C. Lo, Cheuk-Kwong Lee, Kwok-Yung Yuen

Japanese encephalitis virus (JEV) is a mosquitoborne virus endemic to China and Southeast Asia that causes severe encephalitis in <1% of infected persons. Transmission of JEV via blood transfusion has not been reported. We report transmission of JEV via blood donation products from an asymptomatic viremic donor to 2 immunocompromised recipients. One recipient on high-dose immunosuppressive drugs received JEV-positive packed red blood cells after a double lung transplant; severe encephalitis and a poor clinical outcome resulted. JEV RNA was detected in serum, cerebrospinal fluid, and bronchoalveolar lavage fluid specimens. The second recipient had leukemia and received platelets after undergoing chemotherapy. This patient was asymptomatic; JEV infection was confirmed in this person by IgM seroconversion. This study illustrates that, consistent with other pathogenic flaviviruses, JEV can be transmitted via blood products. Targeted donor screening and pathogen reduction technologies could be used to prevent transfusion-transmitted JEV infection in highly JEV-endemic areas.

Japanese encephalitis virus (JEV) is a member of the genus *Flavivirus* and is the eponymous member of the JEV antigenic complex of viruses that also includes West Nile virus (WNV). JEV is endemic to Southeast Asia and China, where ≈67,900 cases occur every year (*1*). The virus is maintained in a complex enzootic cycle involving pigs and birds; humans are infected via the bite of infected *Culex* spp. mosquitoes (particularly *C. tritaeniorhynchus*) (*2*). Humans infected with JEV have low viremia levels and are considered to be dead-end hosts (*3*). Although neurologic manifestations are observed in <1% of infected patients, encephalitis caused by JEV is a devastating condition with a mortality rate of 20%–30%. Survivors often suffer permanent neurologic sequelae. No treatment has been proven to be effective for Japanese encephalitis in clinical trials (*4*).

Arthropodborne viruses are an emerging threat to the blood supply. Transmission via blood transfusion has been described for 4 arthropodborne viruses: dengue virus, WNV, tick-borne encephalitis virus, and Zika virus (*5–10*). Furthermore, chikungunya virus and Usutu virus have also been found in blood donor samples, indicating a risk for transmission via this route as well (*11,12*). In contrast, transfusion-related JEV transmission has not been reported in the literature, although the potential for this type of transmission has been recognized (*13*). In this study, we describe a case of nosocomial Japanese encephalitis in an immunocompromised lung transplant recipient. An outbreak investigation was conducted to ascertain the source of the infection and if other patients were at risk for Japanese encephalitis.

Materials and Methods

Setting

Queen Mary Hospital is a 1,700-bed, university-affiliated, tertiary referral center in Hong Kong that has a lung transplantation service. After lung transplantation, patients are transferred for extended care to Grantham Hospital, a 388-bed specialized respiratory care hospital in the healthcare network of Hong Kong West Cluster.

Virologic Investigations

Virologic investigations for JEV were performed at the Public Health Laboratory Services Branch, Centre for Health Protection, of the Hong Kong Department of Health and the microbiology laboratory at Queen Mary Hospital. We performed JEV IgM testing on serum and cerebrospinal fluid (CSF) using the JE Detect IgM Antibody Capture ELISA (InBiOS, Seattle, WA, USA). We amplified flavivirus RNA in clinical specimens using a conventional

Author affiliations: University of Hong Kong, Hong Kong (V.C.C. Cheng, S. Sridhar, S.C.Y. Wong, J.F.W. Chan, C.C.Y. Yip, K.-Y. Yuen); Queen Mary Hospital, Hong Kong (V.C.C. Cheng, S.-C. Wong, T.W.K. Au, Y.-Y. Hwang); Grantham Hospital, Hong Kong (C.-H. Chau); Centre for Health Protection, Hong Kong (C.S.W. Yau, J.Y.C. Lo); Hong Kong Red Cross Blood Transfusion Service, Hong Kong (C.-K. Lee)

DOI: https://doi.org/10.3201/eid2401.171297

[1]These authors contributed equally to this article.

panflavivirus heminested reverse transcription PCR (RT-PCR) with primers targeting the nonstructural protein 5 (NS5) gene as described previously (*14*). We detected RT-PCR products using gel electrophoresis after the first and second rounds of amplification. We performed real-time RT-PCR specific for JEV using an in-house–developed assay with primers targeting the NS5 gene (forward primer 5′-GGAGCTGGATGGAATGTGAA-3′, reverse primer 5′-TCCCTCCGATGGAAGTATAGAA-3′, probe 6-FAM-CCAAAGCGTATGCACAGATGTGGC-BBQ-650; online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/24/1/17-1297-Techapp1.pdf). We tested for other pathogens that can cause encephalitis, including herpes simplex virus, varicella zoster virus, enterovirus, adenovirus, cytomegalovirus, erythroparvovirus B19, rabies virus, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, and *Toxoplasma gondii*, using in-house–developed PCR and RT-PCR assays (*15*).

Sequencing and Phylogenetic Analysis

Using the inner primer pairs of the panflavivirus heminested RT-PCR, we performed Sanger sequencing of flavivirus NS5 gene amplicons with the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We compared the resulting sequences to the sequences in GenBank using BLAST (*16*) and performed phylogenetic analysis using MEGA 6.06 (http://www.megasoftware.net/). We performed multiple alignment with the NS5 gene sequences obtained from this study (167 nt long) and those of other JEV strains using ClustalW, followed by phylogenetic tree construction using a Kimura 2-parameter substitution model plus invariant site and the maximum-likelihood method as previously described (*17–19*).

Case Definitions

Patients were defined as having JEV infection if any 1 of 2 laboratory criteria was met: detection of JEV-specific IgM in CSF or serum specimens, or detection of JEV RNA in blood or CSF specimens. Patients were defined as having a confirmed transfusion-transmitted JEV infection if they met the above laboratory criteria for JEV infection and received a blood product transfusion from a donor with JEV viremia during the 3 weeks before illness onset. We performed phylogenetic analysis to compare the blood donor and recipient JEV sequences whenever sequences were available.

Outbreak Investigation

Clinical details of the index patient, including date of transplantation, dates of blood transfusions, immunosuppressant dosages, and laboratory investigation results, were retrieved from the electronic patient record system. Archived clinical specimens from the index patient, including serum, CSF, bronchoalveolar lavage fluid, feces, urine, and saliva samples, were retrieved from the microbiology laboratory at Queen Mary Hospital and tested using the panflavivirus heminested PCR assay.

We retrieved details on the organ donor and other transplant recipients from the organ donor registry. We retrieved the remaining blood products of persons who donated blood that was transfused into the index patient during the 3 weeks before illness onset from the Hong Kong Red Cross Blood Transfusion Service and tested these samples using the panflavivirus RT-PCR and JEV-specific RT-PCR described previously.

Because the index patient had resided in Grantham Hospital for the entire JEV incubation period, mosquito presence in Grantham Hospital was directly assessed by interviewing healthcare workers, as described in our previous outbreak investigations (*20–23*). *Culex* vector surveillance is performed by the Food and Environmental Hygiene Department in selected areas of Hong Kong. We were able to retrieve the monthly surveillance data from 1 of their surveillance sites near Grantham Hospital (2.5 km away). This study was approved by the institutional review board of the University of Hong Kong and Hospital Authority Hong Kong West Cluster.

Results

Index Patient

A 52-year-old man with advanced chronic obstructive pulmonary disease underwent double lung transplantation on May 10, 2017, in Queen Mary Hospital. After transplantation, the patient's clinical course was complicated by nosocomial pneumonia; *Burkholderia cepacia* and methicillin-resistant *Staphylococcus aureus* were isolated from the patient's sputum specimens. The infection required a prolonged course of broad-spectrum antimicrobial drugs and ventilator support. Antirejection prophylaxis included tacrolimus (0.5 mg/d), mycophenolate mofetil (250 mg 2×/d), and a tapering course of prednisolone. He was transferred multiple times between Queen Mary Hospital and Grantham Hospital (Figure 1). The patient required transfusions of packed red blood cells for anemia on June 20, 22, and 25 (1 unit/d) in Grantham Hospital.

On July 6 (57 days after transplantation and 11–16 days after transfusions), he had a transient maculopapular rash and fever. Blood tests showed leukocyte (8.99×10^9 cells/L), neutrophil (7.17×10^9 cells/L), and lymphocyte (1.18×10^9 cells/L) counts within reference ranges. Plasma sodium decreased to 128 mmol/L, and liver and renal function test results were unremarkable. His conscious level progressively deteriorated, and he developed myoclonic jerks 2 days after the onset of rash. Serial blood tests showed transient lymphopenia (0.44×10^9 cells/L) and a drop in platelet count (33×10^9/L).

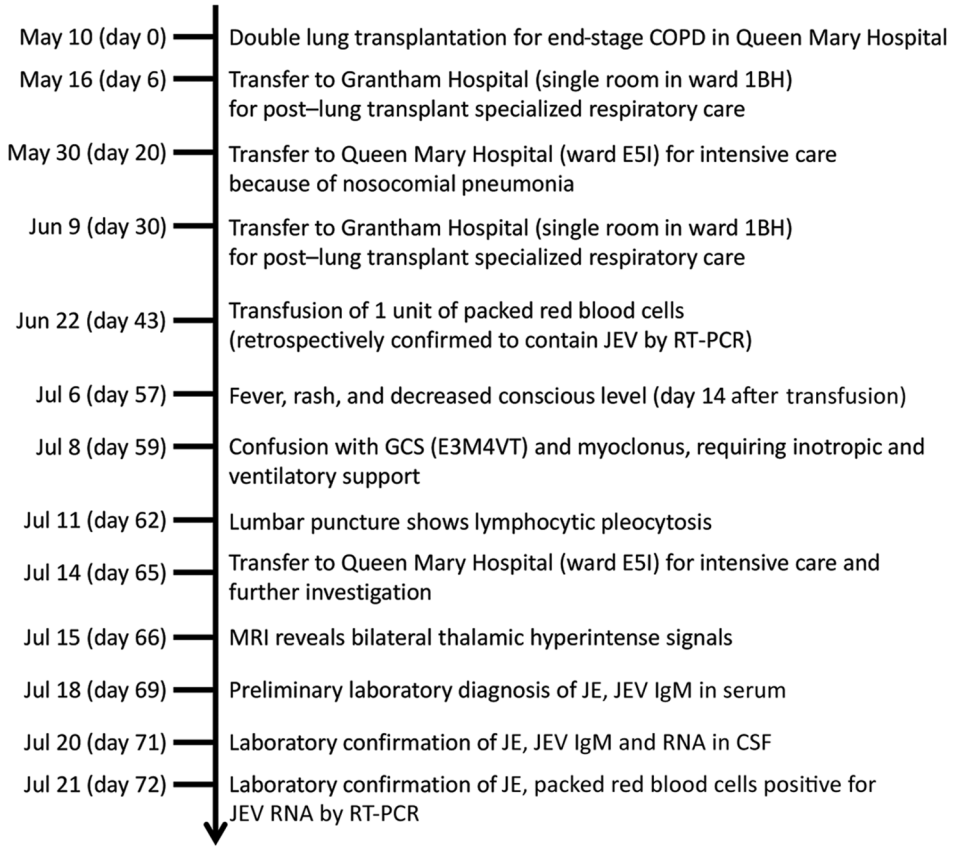


Figure 1. Timeline of index patient with transfusion-transmitted JEV infection, Hong Kong, China, May–July 2017. Day counts indicate the number of days after double lung transplant, unless specified otherwise. COPD, chronic obstructive pulmonary disease; CSF, cerebrospinal fluid; GCS, Glasgow Coma Scale; JEV, Japanese encephalitis virus; MRI, magnetic resonance imaging; RT-PCR, reverse transcription PCR.

A lumbar puncture was performed 6 days after symptom onset. The opening pressure was 13.4 cm H_2O (reference range 6–20 cm H_2O), and the CSF had a total cell count of 20×10^6 cells/L, with a predominance of monocytes (58%) and lymphocytes (35%). CSF protein was elevated (1.61 g/L). Magnetic resonance imaging (MRI) of the brain performed 10 days after symptom onset showed symmetric hyperintensities in the bilateral thalami, substantia nigra, and medial temporal lobes (Figure 2).

In view of the CSF pleocytosis and elevated protein levels, an extensive workup was performed for infectious causes of meningoencephalitis. Gram and Ziehl-Neelsen stains, bacterial and fungal cultures, and the cryptococcal antigen test all gave negative results. PCR for herpes simplex virus, varicella zoster virus, cytomegalovirus, Epstein-Barr virus, enterovirus, rabies virus, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, and *Toxoplasma gondii* were all negative. Serum PCR screening for adenovirus and erythroparvovirus B19 were also negative.

Because of the characteristic distribution of MRI abnormalities involving the basal ganglia, the possibility of flaviviral encephalitis was considered. The patient's CSF was positive for JEV IgM. Subsequent serum samples collected July 15 and 18 were also positive for JEV IgM, but the archived serum specimen obtained May 31, 2017, was negative (Table). Panflavivirus heminested PCR performed on CSF yielded a positive result, with sequencing of the amplicon confirming the presence of JEV (genotype 1). Archived clinical specimens obtained from the patient during the 2 weeks before symptom onset were retrospectively tested using the panflavivirus heminested RT-PCR. Two serum specimens collected June 26 and 28 (10 and 8 days, respectively, before symptom onset) and a bronchoalveolar lavage fluid specimen collected day 5 after symptom onset also tested positive for flavivirus RNA and were confirmed to be JEV by sequencing (GenBank accession no. MF594404). The patient died on October 1, 2017.

Outbreak Investigation

In response to this case of nosocomial acquisition of JEV, the hospital infection control team launched an outbreak investigation in Queen Mary Hospital and Grantham Hospital by tracing the placement of the patient during his entire hospitalization. The index patient was treated in air-conditioned rooms in Queen Mary Hospital and Grantham Hospital throughout his hospitalization. Therefore, mosquitoborne transmission was considered unlikely. Furthermore, a vector surveillance point surveyed by the Food and Environmental Hygiene Department, located 2.5 km from Grantham Hospital, showed that *C. tritaeniorhynchus*

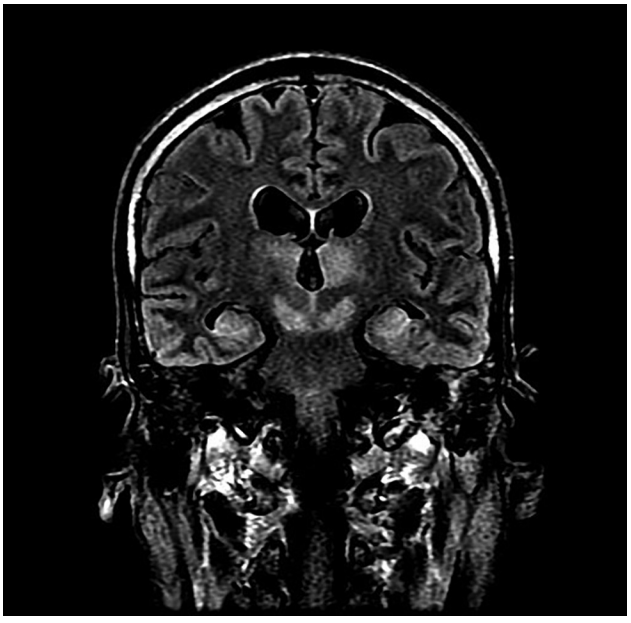


Figure 2. Magnetic resonance imaging of brain of index patient 66 days after double lung transplantation, Hong Kong, China. Coronal FLAIR (FLuid Attenuation Inversion Recovery sequence) image of the head at the level of the lateral ventricles, thalamus, and midbrain shows high signal at bilateral thalamus, midbrain, and medial temporal lobes.

mosquitoes had not been detected since April 2017. Therefore, alternative sources of infection, including blood transfusion, sharps injury with contaminated blood, and organ transplantation, were considered.

The onset of symptoms in the index patient was 57 days after the transplantation date, longer than the usual 5–15-day incubation period for JEV. The organ donor was a 70-year-old road traffic accident victim. Urgent contact tracing of the recipients who received organs (1 liver and 2 kidneys) from this donor revealed that the recipients were asymptomatic. JEV IgM testing of serum samples from these transplant recipients on day 71 (for the liver transplant recipient), day 76 (for renal transplant recipient 1), and day 83 (for renal transplant recipient 2) after transplantation were all negative. A serum sample from the organ donor was not available for JEV testing.

Because the index patient had received 3 units of packed red blood cells from 3 different donors on 3 different days during the 3 weeks before illness onset (i.e., 1 unit each on June 20, June 22, and June 25), the remaining blood products from all 3 donors were traced from the Hong Kong Red Cross Blood Transfusion Service and samples were taken. Only the sample from the transfusion on June 22 tested positive by both real-time RT-PCR for JEV and conventional heminested PCR for flavivirus. Sequencing of the amplicons obtained by conventional PCR yielded a sequence identical to that obtained from the index patient (Figure 3). Phylogenetic comparison with other JEV sequences from GenBank showed that the index case and blood donor isolates belonged to JEV genotype 1, a circulating genotype common in southern China (*24*). The blood donor was a 46-year-old man who resided in Tin Shui Wai of the Yuen Long District in the New Territories region of Hong Kong. He had been asymptomatic at the time of blood donation (May 29, 2017) and had no recent travel to JEV-endemic regions outside of Hong Kong. He did not develop any symptoms of Japanese encephalitis after the blood donation. On July 22, a serum sample from the donor tested positive for JEV IgM. He was deferred from donating blood for 1 year.

Evaluation for Secondary Cases

After confirmation that a blood donor was the source of JEV infection in the index patient, we urgently traced other patients who received blood products from this donor and identified 2 recipients (Table). One was a 61-year-old man with newly diagnosed acute myeloid leukemia who had received induction chemotherapy with cytarabine and daunorubicin during May 8–14, 2017; he was transfused with platelets obtained from the viremic blood donor on June 2, 2017. His white blood cell count on the day of platelet transfusion was 3.39×10^9 cells/L, and he had severe lymphopenia (0.24×10^9 cells/L). His lymphocyte counts steadily rose to 0.95×10^9 cells/L 10 days after the transfusion and normalized by the end of June. He was asymptomatic at follow-up 2 months after transfusion. Upon recognition that this patient received a potentially JEV-infected blood product, staff retrieved his archived serum specimens collected before transfusion and 33 and 50 days after transfusion to test for JEV IgM. The serum specimens collected before transfusion tested negative for JEV IgM, but those collected after tested positive, confirming a recent asymptomatic infection probably contracted from the platelet transfusion. JEV RNA was not detected in a urine specimen collected 50 days after or a plasma sample collected 53 days after the transfusion.

The other blood product recipient was a 64-year-old man who was admitted for intracranial hemorrhage. He received a plasma transfusion June 20, 2017, and died July 4, 2017, due to respiratory failure. No serum specimens were available for serologic testing from this patient, and an autopsy was not performed.

Discussion

In our study, we report nosocomial transmission of JEV through blood transfusion products from an asymptomatic viremic donor to 2 immunocompromised persons, resulting in 1 case of severe encephalitis and another asymptomatic infection with seroconversion. JEV has been documented to be exclusively transmitted via the bite of *Culex* mosquitoes. However, when we diagnosed JEV infection in a

Table. Demographics, investigation results, and clinical details of asymptomatic JEV-infected blood donor and recipients, Hong Kong, China, May–July 2017*

Variable	Donor	Packed red blood cell recipient (index patient)	Platelet recipient	Plasma recipient
Underlying disease	No history of disease, no recent travel to JEV-endemic regions outside Hong Kong; resides in JEV-endemic area Tin Shui Wai, Yuen Long	End-stage chronic obstructive pulmonary disease with lung transplantation, May 10	Acute myeloid leukemia postinduction chemotherapy with cytarabine and daunorubicin	Intracranial hemorrhage
Blood donation or transfusion type, date	Blood donation, May 29	Packed red blood cell transfusion, June 22	Platelet transfusion, June 2	Plasma transfusion, June 20
Pretransfusion IgM serology result, date	Specimen not available	Serum negative, May 31	Serum negative, May 25	Specimen not available
Posttransfusion IgM serology result, date	Serum positive, July 22	CSF positive, July 11; serum positive, July 15 and 18	Serum positive, July 5 and 22	Specimen not available
JEV nucleic acid test, specimen type and result, date	Positive archived blood specimen	Positive blood sample, June 26 and 28; positive bronchoalveolar lavage, July 10	Negative plasma sample, July 22; negative urine sample, July 25	Specimen not available
Clinical symptoms	Asymptomatic	Fever and rash July 6 (14 d after transfusion), followed by decreased consciousness and myoclonic jerks; MRI showed typical appearance of T2 hyperintensity of bilateral thalami, substantia nigra, and medial temporal lobes	Asymptomatic	Unknown
Outcome	Full recovery	Died October 1, 2017	Full recovery	Died from respiratory failure 14 d after transfusion

*CSF, cerebrospinal fluid; JEV, Japanese encephalitis virus; MRI, magnetic resonance imaging.

patient who had been hospitalized for a prolonged period of time, mosquitoborne transmission was considered unlikely because of several factors. First, the index patient was treated in an air-conditioned facility with closed doors and windows during the entire incubation period. Second, rigorous integrated pest management programs (including weekly inspections by dedicated staff and regular monthly pest control services) were implemented in public hospitals and clinics in Hong Kong in 2014 in response to the increasing threat of mosquitoborne infectious diseases, such as dengue in nearby Guangzhou Province, China (*25*). Furthermore, although real-time information on *Culex* spp. breeding density in the district was not available, a *C. tritaeniorhynchus* mosquito distribution survey in Hong Kong showed that adult vector prevalence was low in the Aberdeen area, where Grantham Hospital is located (*26*).

Because of these circumstances, other sources of infection were considered. Transmission via organ transplantation, which has been reported for WNV, was considered (*27*), but serologic screening of other organ recipients from the same donor did not reveal any evidence of JEV infection. Blood product transfusion was then considered as a possible source of infection. JEV RNA was detected in a blood donation sample from 1 donor, and phylogenetic analysis showed 100% sequence identity between the isolates in the donor and recipient, confirming transfusion-transmitted JEV.

The blood donor resided in Tin Shui Wai of the Yuen Long District, an area that has been shown to have a high *C. tritaeniorhynchus* vector density (*26*). From January 2003 through June 2017, seventeen cases of locally acquired JEV infection in Hong Kong were recorded by the Department of Health, and 11 (64.7%) of the 17 patients resided in Yuen Long District. Therefore, the donor was likely to have been infected in Tin Shui Wai. This report illustrates that localized pockets of high JEV endemicity can facilitate the transmission of JEV via unconventional routes.

Arboviruses pose unique threats to the blood supply (*28–30*). Compromise of the blood supply by arboviruses has been well documented during explosive outbreaks of Zika, dengue, WNV, and chikungunya virus infections in recent years (*11,31*). Our findings have major implications for JEV-endemic areas, where undetected transmission of JEV by blood transfusion might be widespread because of high rates of asymptomatic infection in both donors and recipients. In the case we report, 24 days elapsed between the time of blood donation and transfusion to the index patient, indicating that the virus can remain viable in packed red blood cells over a prolonged period of time. Risk assessment of the presence of JEV in the blood supply in JEV-endemic areas should be undertaken.

Mitigation of such transmission is difficult because standard measures to protect the blood supply during

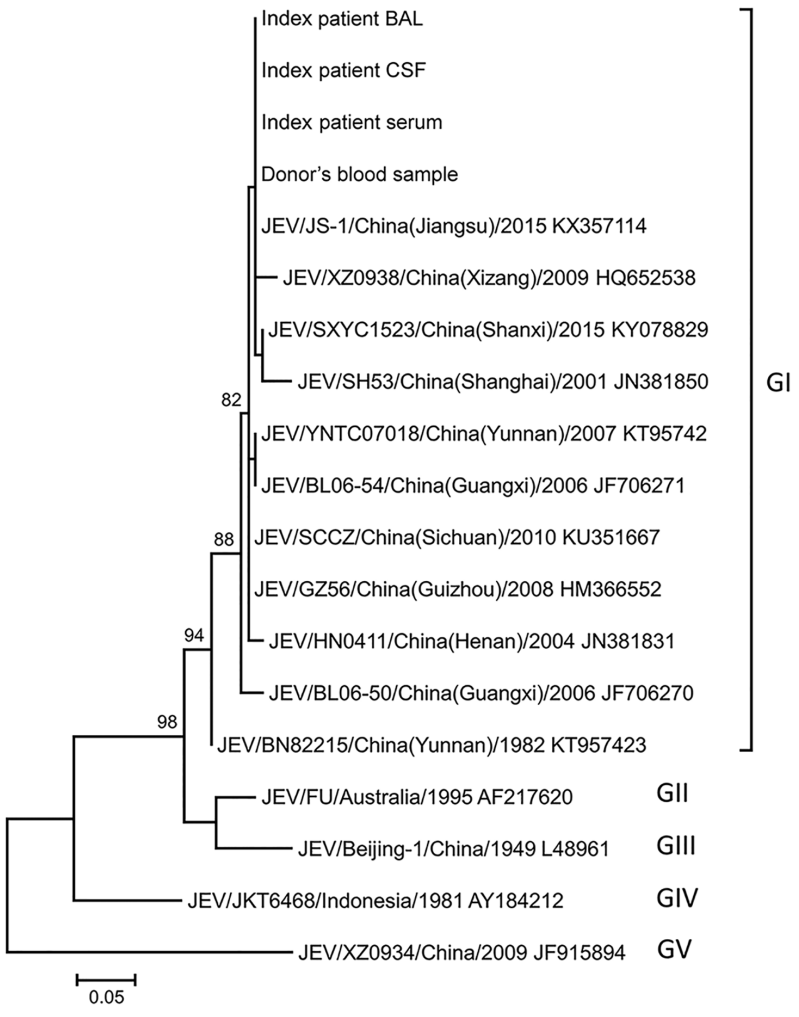


Figure 3. Phylogenetic tree constructed by using partial nonstructural protein 5 (NS5) sequences of JEV isolates detected in index patient and donor blood samples, Hong Kong, China, and other JEV reference strains available in GenBank (accession numbers shown). The tree was inferred from data by using the maximum-likelihood method with bootstrap values calculated from 1,000 trees. Only bootstrap values >70% are shown. A 167-nt fragment of NS5 from each virus was used in this analysis. Labels at right indicate JEV genotypes (GI–V): JEV from patient and donor samples grouped with GI strains. Scale bar indicates estimated number of nucleotide substitutions per 20 nt. BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; JEV, Japanese encephalitis virus.

arboviral outbreaks, such as donor symptom and travel questionnaires (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/1/17-1297-Techapp2.pdf), are not useful in the case of JEV, which has a high rate of subclinical infection and low incidence in Hong Kong. Two approaches to minimize the risk for transfusion-transmitted JEV infection are donor JEV screening and pathogen reduction technology (PRT). Both approaches have limitations. Donor screening would involve nucleic acid testing (NAT) of either individual donors or multiple donors by using minipools, as has been described for WNV and Zika virus (*32–34*). The biggest challenge for implementing screening is the lack of well-established and licensed JEV NAT for use in the blood donor setting. The use of clinical diagnostic NAT for donation screening is associated with the potential for false-positive results by cross-contamination caused by amplicon carryover. Individual donor NAT is likely to be prohibitively expensive, and minipool NAT is difficult to standardize for JEV, given the lack of data on viral loads in asymptomatic persons. Although historically most Japanese encephalitis cases in Hong Kong have occurred during the rainy summer season (May–August), even limited seasonal screening of residents in high-risk areas of Hong Kong might be impractical because of the highly mobile population and imprecise delineation of seasons due to climate change. Although donor screening might not be cost-effective for universal application, a more selective application of blood products used for immunocompromised persons might be considered. However, JEV can cause life-threatening disease even in immunocompetent persons, and the correlation between immunosuppression and disease severity is not clear. A range of novel PRTs for blood products that involve the combined use of ultraviolet light and reagents such as psoralens or riboflavin are available (*35,36*). Examples include the INTERCEPT Blood System for platelets and plasma (Cerus Corporation, Concord, CA, USA); Mirasol Pathogen Reduction Technology for plasma, platelets, and whole blood (Terumo BCT, Lakewood, CO, USA); and the THERAFLEX platform for

plasma and platelets (Macopharma, Tourcoing, France). These methods require individual component processing, are not suitable for all blood components, and are expensive to implement. No direct evidence indicates that these products are efficient at reducing JEV infectivity, although such efficacy could be extrapolated from studies on PRT for Zika and dengue viruses (*37,38*). Also, leukocyte depletion of blood products might theoretically reduce the risk for JEV transmission, but this method also requires validation. Implementation of a JEV vaccination program with high coverage in areas with high *C. tritaeniorhynchus* mosquito breeding density might also be considered to definitively eliminate this virus from the blood supply. If transfusion-transmitted JEV is confirmed to be a significant threat to the blood supply in highly endemic regions, a combination of these methods might be required to prevent transfusion-transmitted JEV infection.

Transmission of WNV, another member of the JEV antigenic serocomplex, to immunocompromised patients via blood transfusion has been reported (*7*). Immunocompromised patients infected with WNV tended to have longer incubation periods (≥10 days) and higher rates of severe illness (*39*). In our study, the index patient had symptom onset 14 days after the transfusion and overt encephalitis 2 days later, a relatively long incubation period, comparable with the observations made for WNV. Clinical specimens from immunocompetent patients with JEV infection are typically PCR negative at symptom onset, reflecting immune-mediated pathogenesis of the disease. However, in our immunocompromised patient, JEV RNA was detected in a serum specimen from 10 days before symptom onset and in bronchoalveolar fluid and CSF samples from days 5 and 6, respectively, after symptom onset, reflecting the inability of this immunocompromised patient to effectively clear the virus. The detection of JEV in lower respiratory tract specimens has not been reported previously. However, WNV has been reported to cause pneumonia in immunocompromised transplant recipients (*40*). The clinical significance of JEV in the respiratory tract of our patient is unclear; the patient had extensive consolidative changes over both lung fields on chest radiograph, but culture of the bronchoalveolar fluid specimen yielded *B. cepacia*, suggesting a component of bacterial pneumonia. However, pulmonary involvement caused by disseminated JEV infection in this immunocompromised patient cannot be excluded.

We identified a second patient who had evidence of JEV seroconversion after receiving platelets from the viremic donor. This patient was immunocompromised at the time of platelet transfusion, which was 19 days after completion of induction chemotherapy for acute leukemia. His relatively asymptomatic clinical course was probably related to the recovery of bone marrow function during the incubation period, in contrast to the index patient, who was subjected to continuous lymphocyte-depleting immunosuppression from the antirejection prophylaxis administered during the incubation period. However, other factors might be responsible, including differences in virus inoculum (the JEV envelope protein has hemagglutinating properties, which could have resulted in a higher accumulation of virus in the packed red blood cell packet); blood product storage conditions (platelet concentrates are stored at higher temperatures, which could have lowered JEV viability in this blood product); and other subtle differences in host-pathogen interactions.

In summary, this study illustrates that JEV can be transmitted via transfusion of cellular blood components to immunocompromised persons and could cause severe outcomes. Enhanced understanding of the prevalence of JEV in the blood supply, the incidence of transfusion-transmitted JEV, and measures for risk mitigation in JEV-endemic areas are urgently needed.

This work was supported in part by donations from Michael Tong, Providence Foundation Ltd (in memory of the late Lui Hac Minh), and the Hong Kong Hainan Commercial Association and by funding from the Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Diseases of the Department of Health, Hong Kong Special Administrative Region, and the Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, the Ministry of Education of China.

Dr. Cheng is a consultant clinical microbiologist and infection control officer at Queen Mary Hospital, Hospital Authority, Hong Kong Special Administrative Region, and honorary professor at the Department of Microbiology at the University of Hong Kong. His major research interests include infection control and clinical infectious diseases. Dr. Sridhar is a clinical assistant professor at the Department of Microbiology of the University of Hong Kong. His major research interests include clinical virology and emerging viruses.

References

1. Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, et al. Estimated global incidence of Japanese encephalitis: a systematic review. Bull World Health Organ. 2011;89:766–74, 774A–774E. http://dx.doi.org/10.2471/BLT.10.085233
2. Chan JF, To KK, Chen H, Yuen KY. Cross-species transmission and emergence of novel viruses from birds. Curr Opin Virol. 2015;10:63–9. http://dx.doi.org/10.1016/j.coviro.2015.01.006
3. Lord JS, Gurley ES, Pulliam JR. Rethinking Japanese encephalitis virus transmission: a framework for implicating host and vector species. PLoS Negl Trop Dis. 2015;9:e0004074. http://dx.doi.org/10.1371/journal.pntd.0004074
4. Chan JF, Chik KK, Yuan S, Yip CC, Zhu Z, Tee KM, et al. Novel antiviral activity and mechanism of bromocriptine as a Zika virus NS2B-NS3 protease inhibitor. Antiviral Res. 2017;141:29–37. http://dx.doi.org/10.1016/j.antiviral.2017.02.002

5. Barjas-Castro ML, Angerami RN, Cunha MS, Suzuki A, Nogueira JS, Rocco IM, et al. Probable transfusion-transmitted Zika virus in Brazil. Transfusion. 2016;56:1684–8. http://dx.doi.org/10.1111/trf.13681
6. Motta IJ, Spencer BR, Cordeiro da Silva SG, Arruda MB, Dobbin JA, Gonzaga YB, et al. Evidence for transmission of Zika virus by platelet transfusion. N Engl J Med. 2016;375:1101–3. http://dx.doi.org/10.1056/NEJMc1607262
7. Pealer LN, Marfin AA, Petersen LR, Lanciotti RS, Page PL, Stramer SL, et al.; West Nile Virus Transmission Investigation Team. Transmission of West Nile virus through blood transfusion in the United States in 2002. N Engl J Med. 2003;349:1236–45. http://dx.doi.org/10.1056/NEJMoa030969
8. Tambyah PA, Koay ES, Poon ML, Lin RV, Ong BK; Transfusion-Transmitted Dengue Infection Study Group. Dengue hemorrhagic fever transmitted by blood transfusion. N Engl J Med. 2008;359:1526–7. http://dx.doi.org/10.1056/NEJMc0708673
9. Chuang V, Wong TY, Leung YH, Ma E, Law YL, Tsang O, et al. Review of dengue fever cases in Hong Kong during 1998 to 2005. Hong Kong Med J. 2008;14:170–7.
10. Wahlberg P, Saikku P, Brummer-Korvenkontio M. Tick-borne viral encephalitis in Finland. The clinical features of Kumlinge disease during 1959–1987. J Intern Med. 1989;225:173–7. http://dx.doi.org/10.1111/j.1365-2796.1989.tb00059.x
11. Simmons G, Brès V, Lu K, Liss NM, Brambilla DJ, Ryff KR, et al. High incidence of chikungunya virus and frequency of viremic blood donations during epidemic, Puerto Rico, USA, 2014. Emerg Infect Dis. 2016;22:1221–8. http://dx.doi.org/10.3201/eid2207.160116
12. Cadar D, Maier P, Müller S, Kress J, Chudy M, Bialonski A, et al. Blood donor screening for West Nile virus (WNV) revealed acute Usutu virus (USUV) infection, Germany, September 2016. Euro Surveill. 2017;22:30501. http://dx.doi.org/10.2807/1560-7917.ES.2017.22.14.30501
13. Dunstan RA, Seed CR, Keller AJ. Emerging viral threats to the Australian blood supply. Aust N Z J Public Health. 2008;32:354–60. http://dx.doi.org/10.1111/j.1753-6405.2008.00254.x
14. Scaramozzino N, Crance JM, Jouan A, DeBriel DA, Stoll F, Garin D. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. J Clin Microbiol. 2001;39:1922–7. http://dx.doi.org/10.1128/JCM.39.5.1922-1927.2001
15. To KK, Tse H, Chan WM, Choi GK, Zhang AJ, Sridhar S, et al. A novel psittacine adenovirus identified during an outbreak of avian chlamydiosis and human psittacosis: zoonosis associated with virus-bacterium coinfection in birds. PLoS Negl Trop Dis. 2014;8:e3318. http://dx.doi.org/10.1371/journal.pntd.0003318
16. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10. http://dx.doi.org/10.1016/S0022-2836(05)80360-2
17. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80. http://dx.doi.org/10.1093/nar/22.22.4673
18. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16:111–20. http://dx.doi.org/10.1007/BF01731581
19. Zhu Z, Chan JF, Tee KM, Choi GK, Lau SK, Woo PC, et al. Comparative genomic analysis of pre-epidemic and epidemic Zika virus strains for virological factors potentially associated with the rapidly expanding epidemic. Emerg Microbes Infect. 2016;5:e22. http://dx.doi.org/10.1038/emi.2016.48
20. Cheng VC, Chan JF, Ngan AH, To KK, Leung SY, Tsoi HW, et al. Outbreak of intestinal infection due to Rhizopus microsporus. J Clin Microbiol. 2009;47:2834–43. http://dx.doi.org/10.1128/JCM.00908-09
21. Cheng VC, Lo WK, Woo PC, Chan SB, Cheng SW, Ho M, et al. Polymicrobial outbreak of intermittent peritoneal dialysis peritonitis during external wall renovation at a dialysis center. Perit Dial Int. 2001;21:296–301.
22. Cheng VC, Wong SS, Chen JH, Chan JF, To KK, Poon RW, et al. An unprecedented outbreak investigation for nosocomial and community-acquired legionellosis in Hong Kong. Chin Med J (Engl). 2012;125:4283–90.
23. Cheng VCC, Chen JHK, Wong SCY, Leung SSM, So SYC, Lung DC, et al. Hospital outbreak of pulmonary and cutaneous zygomycosis due to contaminated linen items from substandard laundry. Clin Infect Dis. 2016;62:714–21. http://dx.doi.org/10.1093/cid/civ1006
24. Chen SP. Molecular phylogenetic and evolutionary analysis of Japanese encephalitis virus in China. Epidemiol Infect. 2012;140:1637–43. http://dx.doi.org/10.1017/S095026881100255X
25. Yang L, Chen Y, Yan H, Zhang P, Xu X, Tang B, et al. A survey of the 2014 dengue fever epidemic in Guangzhou, China. Emerg Microbes Infect. 2015;4:e57. http://dx.doi.org/10.1038/emi.2015.57
26. Food and Environmental Hygiene Department Hong Kong. Distribution of Culex tritaeniorhynchus (JE vector survey 10/04-10/05) [2017 Aug 2]. http://www.fehd.gov.hk/english/safefood/dengue_fever/je_before.pdf
27. Iwamoto M, Jernigan DB, Guasch A, Trepka MJ, Blackmore CG, Hellinger WC, et al.; West Nile Virus in Transplant Recipients Investigation Team. Transmission of West Nile virus from an organ donor to four transplant recipients. N Engl J Med. 2003;348:2196–203. http://dx.doi.org/10.1056/NEJMoa022987
28. Faddy HM, Seed CR, Fryk JJ, Hyland CA, Ritchie SA, Taylor CT, et al. Implications of dengue outbreaks for blood supply, Australia. Emerg Infect Dis. 2013;19:787–9. http://dx.doi.org/10.3201/eid1905.121664
29. Wilder-Smith A, Chen LH, Massad E, Wilson ME. Threat of dengue to blood safety in dengue-endemic countries. Emerg Infect Dis. 2009;15:8–11. http://dx.doi.org/10.3201/eid1501.071097
30. Chan JF, Choi GK, Yip CC, Cheng VC, Yuen KY. Zika fever and congenital Zika syndrome: an unexpected emerging arboviral disease. J Infect. 2016;72:507–24. http://dx.doi.org/10.1016/j.jinf.2016.02.011
31. Petersen LR, Busch MP. Transfusion-transmitted arboviruses. Vox Sang. 2010;98:495–503. http://dx.doi.org/10.1111/j.1423-0410.2009.01286.x
32. Busch MP, Wright DJ, Custer B, Tobler LH, Stramer SL, Kleinman SH, et al. West Nile virus infections projected from blood donor screening data, United States, 2003. Emerg Infect Dis. 2006;12:395–402. http://dx.doi.org/10.3201/eid1205.051287
33. Busch MP, Caglioti S, Robertson EF, McAuley JD, Tobler LH, Kamel H, et al. Screening the blood supply for West Nile virus RNA by nucleic acid amplification testing. N Engl J Med. 2005;353:460–7. http://dx.doi.org/10.1056/NEJMoa044029
34. Ellingson KD, Sapiano MRP, Haass KA, Savinkina AA, Baker ML, Henry RA, et al. Cost projections for implementation of safety interventions to prevent transfusion-transmitted Zika virus infection in the United States. Transfusion. 2017;57 (Suppl 2):1625–33. http://dx.doi.org/10.1111/trf.14164
35. Drew VJ, Barro L, Seghatchian J, Burnouf T. Towards pathogen inactivation of red blood cells and whole blood targeting viral DNA/RNA: design, technologies, and future prospects for developing countries. Blood Transfus. 2017 Apr 13 [Epub ahead of print].

36. Schlenke P. Pathogen inactivation technologies for cellular blood components: an update. Transfus Med Hemother. 2014;41:309–25. http://dx.doi.org/10.1159/000365646
37. Fryk JJ, Marks DC, Hobson-Peters J, Watterson D, Hall RA, Young PR, et al. Reduction of Zika virus infectivity in platelet concentrates after treatment with ultraviolet C light and in plasma after treatment with methylene blue and visible light. Transfusion. 2017. http://dx.doi.org/10.1111/trf.14256
38. Faddy HM, Fryk JJ, Watterson D, Young PR, Modhiran N, Muller DA, et al. Riboflavin and ultraviolet light: impact on dengue virus infectivity. Vox Sang. 2016;111:235–41. http://dx.doi.org/10.1111/vox.12414
39. Levi ME. West Nile virus infection in the immunocompromised patient. Curr Infect Dis Rep. 2013;15:478–85. http://dx.doi.org/10.1007/s11908-013-0367-8
40. Jain N, Fisk D, Sotir M, Kehl KS. West Nile encephalitis, status epilepticus and West Nile pneumonia in a renal transplant patient. Transpl Int. 2007;20:800–3. http://dx.doi.org/10.1111/j.1432-2277.2007.00514.x

Address for correspondence: Kwok-Yung Yuen, Carol Yu Centre for Infection, The University of Hong Kong, University Pathology Building, Queen Mary Hospital Compound, Pokfulam Road, Hong Kong Special Administrative Region, China; email: kyyuen@hku.hk

感染症研究報告　調査報告書

調査報告書番号	KK-kaketsuken-20180219	文献ID	
研究報告の題目	AVIAN INFLUENZA, HUMAN (07): CHINA (JIANGSU) H7N4, FIRST REPORT		

研究報告の概要	報告企業の意見
2018年2月14日，香港のDepartment of Health（衛生部；DH）のCentre for Health Protection（衛生保護センター；CHP）は，ヒトのトリインフルエンザA（H7N4）症例が2018年2月10日～14日に確認されたとNational Health and Family Planning Commission（国家衛生・計画生育委員会；NHFPC）から通知を受け，地元でも旅行中でも，個人衛生，食品衛生，環境衛生を厳重に保持するよう公衆に再度注意喚起した。 NHFPCによると，本件が世界で初めてのヒトのトリインフルエンザA（H7N4）感染症例である。本症例は江蘇省の常州市のLiyangに住む68歳の女性患者で，2017年12月25日に症状を発症した。女性は治療のため2018年1月1日に入院し，2018年1月22日に退院した。女性は症状の発現前に生きた家禽と接触していた。医学的調査期間中，女性と密接に接触したヒトには症状がみられなかった。 Chinese Center for Disease Control and Preventionからの報告によると，分析の結果，本ウイルスの遺伝子はトリが起源であることが明らかになった。 （2018年2月14日付け）	2018年2月14日にWHOから公表された情報によると、中国で世界で初めて人が鳥インフルエンザA（H7N4）患者1人が報告された。これは、世界で初の鳥インフルエンザA（H7N4）が人に感染したことが確認された報告例である。この患者との濃厚接触者は、鳥インフルエンザA（H7N4）に対する検査が陰性であり、症状もみられていないことから、現時点では、このウイルスには人から人への感染伝播能力はないことが示唆され、パンデミックを引き起こす可能性は極めて低いものと考えられる。

今後の対応	その他参考事項等
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。	

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

Published Date: 2018-02-14 16:02:35
Subject: PRO/AH/EDR> Avian influenza, human (07): China (JS) H7N4, 1st report
Archive Number: 20180214.5628629

AVIAN INFLUENZA, HUMAN (07): CHINA (JIANGSU) H7N4, FIRST REPORT
****

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: Wed 14 Feb 2018
Source: Hong Kong Govt. Press Release[edited]
http://www.info.gov.hk/gia/general/201802/14/P2018021400759.htm

The Centre for Health Protection (CHP) of the Department of Health (DH) received today [Wed 14 Feb 2018] a notification from the National Health and Family Planning Commission (NHFPC) that a human case of avian influenza A (H7N4) was confirmed from [10 to 14 Feb 2017], and reminded the public to maintain strict personal, food and environmental hygiene both locally and during travel.

According to the NHFPC, this is the 1st case of human infection with avian influenza A (H7N4) in the world. The case involved a 68-year-old female patient living in Liyang in Changzhou of Jiangsu Province who developed symptoms on [25 Dec 2017]. She was admitted to hospital for medical treatment on [1 Jan 2018] and was discharged on [22 Jan 2018]. She had contact with live poultry before the onset of symptoms. All her close contacts did not have any symptoms during the medical surveillance period.

According to a report from the Chinese Center for Disease Control and Prevention, upon analysis, the genes of the virus were determined to be of avian origin. "All novel influenza A infections, including H7N4, are notifiable infectious diseases in Hong Kong," the spokesman for the CHP said.

"Based on the seasonal pattern, the activity of avian influenza viruses is expected to be higher in winter. Travellers to the Mainland or other affected areas must avoid visiting wet markets, live poultry markets or farms. They should be alert to the presence of backyard poultry when visiting relatives and friends. They should also avoid purchasing live or freshly slaughtered poultry, and avoid touching poultry/birds or their droppings. They should strictly observe personal and hand hygiene when visiting any place with live poultry," the spokesman reminded.

Travellers returning from affected areas should consult a doctor promptly if symptoms develop, and inform the doctor of their travel history for prompt diagnosis and treatment of potential diseases. It is essential to tell the doctor if they have seen any live poultry during travel, which may imply possible exposure to contaminated environments. This will enable the doctor to assess the possibility of avian influenza and arrange necessary investigations and appropriate treatment in a timely manner.

While local surveillance, prevention and control measures are in place, the CHP will remain vigilant and work closely with the World Health Organization and relevant health authorities to monitor the latest developments.

The CHP's Port Health Office conducts health surveillance measures at all boundary control points. Thermal imaging systems are in place for body temperature checks on inbound travellers. Suspected cases will be immediately referred to public hospitals for follow-up.

The display of posters and broadcasting of health messages in departure and arrival halls as health education for travellers is under way. The travel industry and other stakeholders are regularly updated on the latest information.

The public should maintain strict personal, hand, food and environmental hygiene and take heed of the advice below if handling poultry:

- Avoid touching poultry, birds, animals or their droppings;

- When buying live chickens, do not touch them and their droppings. Do not blow at their bottoms. Wash eggs with detergent if soiled with faecal matter and cook and consume the eggs immediately. Always wash hands thoroughly with soap and water after handling chickens and eggs;

- Eggs should be cooked well until the white and yolk become firm. Do not eat raw eggs or dip cooked food into any sauce with raw eggs.

- Poultry should be cooked thoroughly. If there is pinkish juice running from the cooked poultry or the middle part of its bone is still red, the poultry should be cooked again until fully done;

- Wash hands frequently, especially before touching the mouth, nose or eyes, before handling food or eating, and after going to the toilet, 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; and

- Wear a mask if fever or respiratory symptoms develop, when going to a hospital or clinic, or while taking care of patients with fever or respiratory symptoms.

The public may visit the CHP's pages for more information: the avian influenza page, the weekly Avian Influenza Report, global statistics and affected areas of avian influenza, the Facebook Page and the YouTube Channel.

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

[This is the 1st report of a human case infected with avian influenza A/H7N4. This raises concerns in view of the recent emergence of Influenza A/H7N9 viruses that have increasingly caused severe disease in humans since their emergence in 2013. The fact that surveillance in live bird markets and poultry as well as humans has been increased in recent years may have contributed to early detection of this novel influenza A/H7subtype, but it also indicates that not only are these avian viruses evolving continuously; it is evidence of bird to human transmission events that increase the likelihood of emergence of viruses with pandemic potential. - Mod.UBA

HealthMap/ProMED-mail map:
Jiangsu Province, China: http://healthmap.org/promed/p/342]

See Also

Avian influenza, human (06): China (GD) H7N9 20180213.5625277
Avian influenza, human (05): human-animal interface, WHO 20180205.5606724
Avian influenza, human (01): China (FJ) H5N6 20180109.5546265
2017

Avian influenza, human (82): human-animal interface, WHO 20171227.5523467
Avian influenza, human (81): China (YN) H7N9 20171204.5481306
Avian Influenza, human (80): China (GX) H5N6 20171121.5457466
Avian influenza, human (70): China, H7N9 20170911.5306399
Avian influenza, human (60): WHO, human-animal interface Avian influenza, human (50): China, H7N9 20170509.5019418
Avian influenza, human (40): Egypt (FY), H5N1, comments 20170402.4939591
Avian influenza, human (30): China, H7N9 20170307.4885433
Avian influenza, human (20): China (SC, YN, BJ), H7N9, death toll 20170215.4841682
Avian influenza, human (10): Indonesia (LA) RFI 20170123.4785841
Avian influenza, human (01): China (JX), H7N9 20170102.4736553
2016

Avian influenza, human (72): China (HK) H7N9, fatal 20161229.4727495
Avian influenza, human (68): China, H7N9 20161218.4705001
Avian influenza, human (67): WHO, H5N6, H7N9, risk assessment 20161212.4689184
Avian influenza, human (66): China (GD) H7N9 20161210.4689085
Avian influenza, human (65): China (HN) H5N6 20161123.4646005
Avian influenza, human (64): China (ZJ,JS) H7N9 20161114.4624064

Avian influenza, human (60): China (JX) H9N2 20160912.4481431
Avian influenza, human (58): China, H7N9, update, WHO 20160820.4422893
Avian influenza, human (57): China, H7N9, WHO update 20160727.4370565
and other items in the archives
.....uba/ao/lm

Published Date: 2017-11-04 16:21:59
Subject: PRO/AH/EDR> Avian influenza, human (76): Russia, H5N8
Archive Number: 20171104.5419594

AVIAN INFLUENZA, HUMAN (76): RUSSIA, H5N8
****
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 31 Oct 2017 17:07
Source: Russia News [In Russian, translated by Google, edited]
http://news-russia.info/2017/10/31/rukovoditel-rospotrebnadzora-soobschila-o-viyavlenii-u/

We are talking about the virus H5N8, which was previously observed only in birds, warns Rospotrebnadzor. At the same time, as the head of Rospotrebnadzor, Anna Popova reported today [Tue 31 Oct 2017], Russian scientists confirmed the possibility of asymptomatic human infection with the avian influenza H5N8 virus, which was previously observed only in birds. This confirms that the virus has crossed an interspecies barrier and acquired new properties that may be potentially unsafe for humans, RIA Novosti reported referring to the head of Rospotrebnadzor Anna Popova.

However, at the same time, we found that when examining the blood sera of people who were in contact, they took care of birds in these outbreaks. Antibodies to influenza viruses and H5N1 and H5N8 were found in the blood almost without clinical occurrences, hence the interspecies transition threshold. The head of Rospotrebnadzor expressed hope that in the future this strain will not lead to negative results.

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

[According to Popova, for scientists this situation will be a motivation for the continuation of observations and research on avian flu. And outbreaks of influenza in birds in the very beginning of 2016 were registered in 48 countries of Asia, Africa, North America and Europe.

As mentioned in the report, Influenza A/H5N8 has been reported previously only from poultry and wildfowl from over 24 countries in Europe, and parts of Asia, Middle East and Africa, and the A(H5N8) virus has been circulating continuously in Asia since 2010 [https://www.sciencedaily.com/releases/2016/11/161121170044.htm]. According to the WHO assessment 'Human infection with the A(H5N8) virus cannot be excluded, although the likelihood is low, based on the limited information obtained to date. It should be noted that human infection with A(H5N6) of related clade 2.3.4.4 has already occurred'. [http://www.who.int/influenza/human_animal_interface/avian_influenza/riskassessment_AH5N8_201611/en/].

A report from November 2016 concluded 'Full genome sequencing of recent HPAI A(H5N8) viruses suggest that these viruses remain essentially bird viruses without any specific increased risk for humans. No human infections with this virus have ever been reported world-wide. ECDC's updated rapid risk assessment concludes that the risk of transmission to the general public in Europe is considered to be very low'[https://www.sciencedaily.com/releases/2016/11/161121170044.htm]. However, based on the available information of other A(H5) subtype viruses, human population immunity against the recently detected A(H5N8) viruses is expected to be minimal.
Continued surveillance for all highly pathogenic avian influenza A(H5) viruses will be important to monitor their occurrence and evolution. Timely sharing of representative viruses and sequence information is critical for a complete assessment of the risk posed by these viruses.
Public health advice
- Avoid contact with any birds (poultry or wild birds) or other animals that are sick or are found dead and report them to the relevant authorities.
- Wash hands properly with soap or a suitable disinfectant.
- Follow good food safety and good food hygiene practices.

Russia has reported poultry outbreaks with this avian influenza virus since 2014, and this report of a human case mandates a detailed investigation in to underlying factors. - Mod.UBA

A HealthMap/ProMED-mail map can be accessed at: http://healthmap.org/promed/p/98.]

See Also

Avian influenza, human (66): Philippines, H5 20170818.5254521
Avian influenza (152): Cyprus wild bird, HPAI H5N8, OIE, Bulgaria HPAI H5N8 20171024.5401457
Avian influenza (151): Germany wild bird, HPAI H5N8, Bulgaria poultry, H5, OIE 20171023.5398822
Avian influenza (150): Germany (NI) wild bird, HPAI H5N8, regional alarm 20171021.5395412
Avian influenza (146): Italy, north, poultry, wild, HPAI H5N8, spread 20171012.5377180
Avian influenza (133): Germany (ST) wild bird, HPAI H5N8, OIE 20170829.5282824
Avian influenza (129): Switzerland, HPAI H5N8, swan 20170816.5255122
Avian influenza (125): UK (England) wild bird, HPAI H5N8, OIE 20170807.5234463
Avian influenza (121): Belgium (LG) captive birds, HPAI H5N8, spread 20170707.5157930
Avian influenza (110): UK, England poultry, backyard, HPAI H5N8 20170604.5083412
Avian influenza (109): Belgium (LX) phasianidae, HPAI H5N8, OIE 20170603.5081763
Avian influenza (107): Europe (Luxembourg) poultry 20170602.5080342
Avian influenza (99): UK (England) poultry, backyard, HPAI H5N8, OIE 20170508.5020992
Avian influenza (97): UK (England) poultry, backyard, HPAI H5N8 20170506.5016687
Avian influenza, human (33): Indonesia (JR) H5 susp, RFI 20170315.4898106
Avian influenza, human (31): Russia (MS) H5N8 susp, RFI 20170308.4886536
Avian influenza (58): Europe (Belgium, Czech Rep) wildfowl, poultry, spread 20170224.4862423
Avian influenza (49): Ireland (CO, TY) HPAI H5N8, resident wild birds 20170213.4836217
Avian influenza (44): Slovakia, H5N8, poultry, wild birds 20170204.4815929
Avian influenza (40): Portugal (Algarve) wildfowl, HPAI H5N8, OIE 20170202.4811961
Avian influenza (39): Uganda, HPAI H5N8, wildfowl, poultry, FAO, migration concern 20170202.4810412
Avian influenza (35): Greece wildfowl HPAI H5N5 Macedonia poultry HPAI H5N8, OIE 20170130.4803658
Avian influenza (31): Europe, Asia, HPAI H5N8, poultry, wildfowl, spread 20170125.4790548
Avian influenza (30): Italy (VN) poultry, HPAI H5N8, OIE 20170124.4787128
Avian influenza (28): Iran, poultry, H5N8, spread 20170123.4786909
Avian influenza (25): Greece (PW) poultry, HPAI H5N8, OIE 20170119.4775956
Avian influenza (24): China (HU), captive swans, HPAI H5N8, OIE 20170119.4775955
Avian influenza (23): India (GJ) wildfowl, H5N8, OIE 20170119.4775954
Avian influenza (19): Uganda, HPAI H5, 1st rep, comment 20170118.4772970
Avian influenza (18): China (Shanxi) swan, HPAI H5N8 20170118.4772587
Avian influenza (17): Uganda, poultry, wild birds, HPAI H5, 1st report, OIE, RFI 20170117.4771686
Avian influenza (16): Europe, HPAI H5, H5N8, wildfowl 20170117.4771394
Avian influenza (15): Russia (KD) swan, HPAI H5, OIE 20170117.4770239
Avian influenza (14): Ukraine, Slovakia, poultry, captive/wild bird HPAI H5N8, OIE 20170113.4764108
Avian influenza (13): Europe, wild bird, HPAI H5N8, new strain, OIE 20170112.4760583
Avian influenza (11): Ireland (WX) wild bird, HPAI H5N8, new strain, OIE 20170111.4759033
Avian influenza (10): global, H5N8 clade 2.3.4.4, FAO update & recommendations 20170111.4758712
Avian influenza (09): UK (England) HPAI H5N8, swan 20170111.4757028
Avian influenza (05): Europe, Asia, H5N8, poultry, wild, spread 20170109.4752395
Avian influenza (08): Slovenia, HPAI H5N8, swan 20170111.4757155
Avian influenza (05): Europe, Asia, H5N8, poultry, wild, spread 20170109.4752395
Avian influenza (04): Slovakia (NI) HPAI H5, wild bird, 1st rep 20170109.4751637
Avian influenza (03): Europe, Asia, H5N8, poultry, wild, spread 20170106.4745034
Avian influenza (01): Croatia (VP) HPAI H5N8, 1st report 20170101.4736233
Avian influenza, human (09): Uganda, H5 susp, RFI 20170122.4779120

2015

Avian influenza, human (118): CDC H5 virus risk assessment 20150731.3548675
Avian influenza, human (27): WHO update H5 & H7N9, China (GD) H7N9 20150129.3129613
.....sb/uba/ec/lm

Published Date: 2018-02-14 16:02:35
Subject: PRO/AH/EDR> Avian influenza, human (07): China (JS) H7N4, 1st report
Archive Number: 20180214.5628629

AVIAN INFLUENZA, HUMAN (07): CHINA (JIANGSU) H7N4, FIRST REPORT
****
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: Wed 14 Feb 2018
Source: Hong Kong Govt. Press Release[edited]
http://www.info.gov.hk/gia/general/201802/14/P2018021400759.htm

The Centre for Health Protection (CHP) of the Department of Health (DH) received today [Wed 14 Feb 2018] a notification from the National Health and Family Planning Commission (NHFPC) that a human case of avian influenza A (H7N4) was confirmed from [10 to 14 Feb 2017], and reminded the public to maintain strict personal, food and environmental hygiene both locally and during travel.

According to the NHFPC, this is the 1st case of human infection with avian influenza A (H7N4) in the world. The case involved a 68-year-old female patient living in Liyang in Changzhou of Jiangsu Province who developed symptoms on [25 Dec 2017]. She was admitted to hospital for medical treatment on [1 Jan 2018] and was discharged on [22 Jan 2018]. She had contact with live poultry before the onset of symptoms. All her close contacts did not have any symptoms during the medical surveillance period.

According to a report from the Chinese Center for Disease Control and Prevention, upon analysis, the genes of the virus were determined to be of avian origin. "All novel influenza A infections, including H7N4, are notifiable infectious diseases in Hong Kong," the spokesman for the CHP said.

"Based on the seasonal pattern, the activity of avian influenza viruses is expected to be higher in winter. Travellers to the Mainland or other affected areas must avoid visiting wet markets, live poultry markets or farms. They should be alert to the presence of backyard poultry when visiting relatives and friends. They should also avoid purchasing live or freshly slaughtered poultry, and avoid touching poultry/birds or their droppings. They should strictly observe personal and hand hygiene when visiting any place with live poultry," the spokesman reminded.

Travellers returning from affected areas should consult a doctor promptly if symptoms develop, and inform the doctor of their travel history for prompt diagnosis and treatment of potential diseases. It is essential to tell the doctor if they have seen any live poultry during travel, which may imply possible exposure to contaminated environments. This will enable the doctor to assess the possibility of avian influenza and arrange necessary investigations and appropriate treatment in a timely manner.

While local surveillance, prevention and control measures are in place, the CHP will remain vigilant and work closely with the World Health Organization and relevant health authorities to monitor the latest developments.

The CHP's Port Health Office conducts health surveillance measures at all boundary control points. Thermal imaging systems are in place for body temperature checks on inbound travellers. Suspected cases will be immediately referred to public hospitals for follow-up.

The display of posters and broadcasting of health messages in departure and arrival halls as health education for travellers is under way. The travel industry and other stakeholders are regularly updated on the latest information.

The public should maintain strict personal, hand, food and environmental hygiene and take heed of the advice below if handling poultry:

- Avoid touching poultry, birds, animals or their droppings;

- When buying live chickens, do not touch them and their droppings. Do not blow at their bottoms. Wash eggs with detergent if soiled with faecal matter and cook and consume the eggs immediately. Always wash hands thoroughly with soap and water after handling chickens and eggs;

- Eggs should be cooked well until the white and yolk become firm. Do not eat raw eggs or dip cooked food into any sauce with raw eggs.

- Poultry should be cooked thoroughly. If there is pinkish juice running from the cooked poultry or the middle part of its bone is still red, the poultry should be cooked again until fully done;

- Wash hands frequently, especially before touching the mouth, nose or eyes, before handling food or eating, and after going to the toilet, 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; and

- Wear a mask if fever or respiratory symptoms develop, when going to a hospital or clinic, or while taking care of patients with fever or respiratory symptoms.

The public may visit the CHP's pages for more information: the avian influenza page, the weekly Avian Influenza Report, global statistics and affected areas of avian influenza, the Facebook Page and the YouTube Channel.

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

[This is the 1st report of a human case infected with avian influenza A/H7N4. This raises concerns in view of the recent emergence of Influenza A/H7N9 viruses that have increasingly caused severe disease in humans since their emergence in 2013. The fact that surveillance in live bird markets and poultry as well as humans has been increased in recent years may have contributed to early detection of this novel influenza A/H7subtype, but it also indicates that not only are these avian viruses evolving continuously; it is evidence of bird to human transmission events that increase the likelihood of emergence of viruses with pandemic potential. - Mod.UBA

HealthMap/ProMED-mail map:
Jiangsu Province, China: http://healthmap.org/promed/p/342]

See Also

Avian influenza, human (06): China (GD) H7N9 20180213.5625277
Avian influenza, human (05): human-animal interface, WHO 20180205.5606724
Avian influenza, human (01): China (FJ) H5N6 20180109.5546265
2017

Avian influenza, human (82): human-animal interface, WHO 20171227.5523467
Avian influenza, human (81): China (YN) H7N9 20171204.5481306
Avian Influenza, human (80): China (GX) H5N6 20171121.5457466
Avian influenza, human (70): China, H7N9 20170911.5306399
Avian influenza, human (60): WHO, human-animal interface Avian influenza, human (50): China, H7N9 20170509.5019418
Avian influenza, human (40): Egypt (FY), H5N1, comments 20170402.4939591
Avian influenza, human (30): China, H7N9 20170307.4885433
Avian influenza, human (20): China (SC, YN, BJ), H7N9, death toll 20170215.4841682
Avian influenza, human (10): Indonesia (LA) RFI 20170123.4785841
Avian influenza, human (01): China (JX), H7N9 20170102.4736553
2016

Avian influenza, human (72): China (HK) H7N9, fatal 20161229.4727495
Avian influenza, human (68): China, H7N9 20161218.4705001
Avian influenza, human (67): WHO, H5N6, H7N9, risk assessment 20161212.4689184
Avian influenza, human (66): China (GD) H7N9 20161210.4689085
Avian influenza, human (65): China (HN) H5N6 20161123.4646005
Avian influenza, human (64): China (ZJ,JS) H7N9 20161114.4624064

Avian influenza, human (60): China (JX) H9N2 20160912.4481431
Avian influenza, human (58): China, H7N9, update, WHO 20160820.4422893
Avian influenza, human (57): China, H7N9, WHO update 20160727.4370565
and other items in the archives
.....uba/ao/lm

Emergencies preparedness, response

Human infection with avian influenza A (H7N4) virus – China

Disease outbreak news
22 February 2018

On 14 February 2018, the National Health and Family Planning Commission (NHFPC) of China notified the World Health Organization (WHO) of one case of human infection with avian influenza A(H7N4) virus. This is the first human case of avian influenza A(H7N4) infection to be reported worldwide.

The case-patient was a 68-year-old woman from Jiangsu Province with pre-existing coronary heart disease and hypertension and she developed symptoms on 25 December 2017. Seven days later, she was admitted to a local hospital for treatment of severe pneumonia and was discharged after 21 days. On 12 February, the Chinese Center for Disease Control and Prevention (China CDC) confirmed that the case-patient's samples were positive for avian influenza A(H7N4). The NHFPC confirmed the diagnosis on 13 February 2018. The case-patient had reported a history of exposure to live poultry before onset of symptoms.

Genetic sequencing of this A(H7N4) virus shows that all the virus segments originated from avian influenza viruses. This virus is sensitive to adamantanes and neuraminidase inhibitors based on genetic sequencing.

Twenty-eight close contacts of the case-patient have been under medical observation. Among close contacts, no abnormal findings have been found and all throat swabs from her contacts have tested negative.

Public health response

The Chinese government conducted a risk assessment, and has enhanced prevention and control measures, surveillance and epidemiological investigations including contact tracing and laboratory testing. Public risk communication and information sharing is ongoing.

WHO is in contact with national authorities and is following the event closely. WHO is facilitating information-sharing with Member States and is closely monitoring the situation, in line with the International Health Regulations (2005).

WHO risk assessment

This is the first report of a human case of avian influenza A(H7N4) infection globally and the case reported exposure to live backyard poultry

before illness onset. Genetic analysis of this influenza A(H7N4) virus indicates that it is of avian origin.

Close contacts of the case-patient tested negative for avian influenza A (H7N4) and remained asymptomatic. Current evidence suggests that this virus does not have the ability of sustained transmission to humans, thus the likelihood of sustained human to human transmission is low. Any animal influenza virus that develops the ability of human to human transmission can theoretically cause a pandemic.

It is possible that additional human cases of avian influenza A(H7N4) will be detected, however only one human case has been detected so far, and information on the circulation of avian influenza A(H7N4) in birds is not currently available. Further information needs to be gathered to increase the confidence in this assessment.

WHO advice

The public should avoid contact with high-risk environments such as live animal markets/farms and live poultry, or surfaces that might be contaminated by poultry feces. Hand hygiene with frequent washing or use of alcohol hand sanitizer is recommended. WHO does not recommend any specific different measures for travellers.

WHO does not advise special screening at points of entry with regard to this event, nor does it recommend that any travel or trade restrictions be applied.

Related links

Fact sheet on avian influenza

More avian influenza outbreak news

China country profile

Avian and other zoonotic influenza

感染症研究報告　調査報告書

調査報告書番号	KK-BYL-0440	文献ID	
研究報告の題目	Avian Influenza, human (76): Russia, H5N8		

研究報告の概要	報告企業の意見
問題点：ロシアにおいてトリインフルエンザA（H5N8）ウイルスによる初めてのヒト感染例が確認された。 【概要】新たにヒトにおいて感染することが認められた感染症に関する報告。 2017年10月31日、ロシア連邦消費者権利保護福利監督庁長官は、以前はトリでしかみられなかったトリインフルエンザA（H5N8）ウイルスによる無症候性ヒト感染の可能性症例が、ロシアの研究者により確認されたと発表した。これにより、本ウイルスが種間障壁を越え、ヒトにおいて潜在的に安全でない新しい特性を獲得したことが確認された。 しかし同時に、接触した複数のヒトの血清を検査した際、彼らが本ウイルス感染流行中にトリの世話をしていたことが分かった。ほとんど臨床症状もなくそれらの血清からトリインフルエンザA（H5N1）ウイルスおよび本ウイルスの抗体が検出され、それゆえに種間遷移閾値が認められた。	本件は、ロシアにおいてトリインフルエンザA（H5N8）ウイルスによる初めてのヒト感染例が確認された報告である。 コージネイトFSでは、製造工程における病原体除去・不活化処理は、ウイルス及び細菌に対して有効であることが報告されている。また、コバールトリイにおいては、マスターセルバンク及びワーキングセルバンクの凍結培地の一部にヒト血漿タンパク溶液を使用しているが、培養工程以降では使用していない。

今後の対応	その他参考事項等
現時点で新たな安全対策上の措置を講じる必要はないと考える。今後も、新規人畜共通感染症や新たなウイルス感染症に関する情報収集に努める。	

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

Emergencies preparedness, response

Human infection with avian influenza A (H7N4) virus – China

Disease outbreak news
22 February 2018

On 14 February 2018, the National Health and Family Planning Commission (NHFPC) of China notified the World Health Organization (WHO) of one case of human infection with avian influenza A(H7N4) virus. This is the first human case of avian influenza A(H7N4) infection to be reported worldwide.

The case-patient was a 68-year-old woman from Jiangsu Province with pre-existing coronary heart disease and hypertension and she developed symptoms on 25 December 2017. Seven days later, she was admitted to a local hospital for treatment of severe pneumonia and was discharged after 21 days. On 12 February, the Chinese Center for Disease Control and Prevention (China CDC) confirmed that the case-patient's samples were positive for avian influenza A(H7N4). The NHFPC confirmed the diagnosis on 13 February 2018. The case-patient had reported a history of exposure to live poultry before onset of symptoms.

Genetic sequencing of this A(H7N4) virus shows that all the virus segments originated from avian influenza viruses. This virus is sensitive to adamantanes and neuraminidase inhibitors based on genetic sequencing.

Twenty-eight close contacts of the case-patient have been under medical observation. Among close contacts, no abnormal findings have been found and all throat swabs from her contacts have tested negative.

Public health response

The Chinese government conducted a risk assessment, and has enhanced prevention and control measures, surveillance and epidemiological investigations including contact tracing and laboratory testing. Public risk communication and information sharing is ongoing.

WHO is in contact with national authorities and is following the event closely. WHO is facilitating information-sharing with Member States and is closely monitoring the situation, in line with the International Health Regulations (2005).

WHO risk assessment

This is the first report of a human case of avian influenza A(H7N4) infection globally and the case reported exposure to live backyard poultry

before illness onset. Genetic analysis of this influenza A(H7N4) virus indicates that it is of avian origin.

Close contacts of the case-patient tested negative for avian influenza A (H7N4) and remained asymptomatic. Current evidence suggests that this virus does not have the ability of sustained transmission to humans, thus the likelihood of sustained human to human transmission is low. Any animal influenza virus that develops the ability of human to human transmission can theoretically cause a pandemic.

It is possible that additional human cases of avian influenza A(H7N4) will be detected, however only one human case has been detected so far, and information on the circulation of avian influenza A(H7N4) in birds is not currently available. Further information needs to be gathered to increase the confidence in this assessment.

WHO advice

The public should avoid contact with high-risk environments such as live animal markets/farms and live poultry, or surfaces that might be contaminated by poultry feces. Hand hygiene with frequent washing or use of alcohol hand sanitizer is recommended. WHO does not recommend any specific different measures for travellers.

WHO does not advise special screening at points of entry with regard to this event, nor does it recommend that any travel or trade restrictions be applied.

Related links

Fact sheet on avian influenza

More avian influenza outbreak news

China country profile

Avian and other zoonotic influenza

感染症研究報告　調査報告書

調査報告書番号	KK-JRC-000018	文献ID	28867401
研究報告の題目	Outbreak of human malaria caused by Plasmodium simium in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation.		

研究報告の概要	報告企業の意見
○リオデジャネイロ州の大西洋岸森林地域での_Plasmodium simium_(_P.simium_)によるヒトマラリアのアウトブレイク 背景：ブラジル南部及び南東部において、マラリアは50年以上前に排除されたが、リオデジャネイロ州の大西洋岸森林地域で行われた最近の調査により、_Plasmodium vivax_(_P.vivax_：三日熱マラリア原虫)による土着性の症例数の増加が明らかとなった。非ヒト霊長類を宿主とする、_P.vivax_様マラリア原虫である_P.simium_は、同地域のサルにおける風土病の原因であることから、同原虫が人獣共通感染を引き起こすかどうかを調査した。 方法：マラリアの兆候や症状を呈する患者（リオデジャネイロ州の大西洋岸森林地域に滞在歴のある者及び居住者を対象とし、マラリア予防薬を服用した者、輸血や臓器移植患者及び前年にマラリア流行地域に滞在した者は除外）及び現地のホエザルの血液検体に対し、鏡検及びRT-PCR法による検査を実施した。さらに、遺伝子配列により_P.vivax_と_P.simium_を区別するタイピング法を用いて、2015年と2016年に発生した症例のうち33例を解析した。 結果：2015年から2016年に報告された土着性マラリア症例は49例であった。症例の82%はリオデジャネイロ州の都市部に居住し、レジャー又は出張で大西洋岸森林地域を訪れたことがあった。 タイピング法により33例中28例の解析が成功し、全て_P.simium_であったことから、同原虫は、同地域において人獣共通感染の原因となっていることが示唆された。これらの検体のうち3例の全ミトコンドリアゲノム配列は、_P.simium_が南米の_P.vivax_に最も密接に関連していた。 これまで_P.simium_はサルに特異的な原虫で_P.vivax_とは別の種と考えられており、ヒトへの感染は明らかにされていない。 結論：本研究により同原虫が人獣共通感染することが明確に実証された。当該地域において、これまで同原虫のヒトへの感染は常に発生していたが、両原虫を区別する方法が存在しなかったため、_P.vivax_と誤診されていた可能性がある。同原虫の公衆衛生に与える影響とブラジルにおけるマラリアの排除状況を評価するためには、現地の非ヒト霊長類及びハマダラカを対象としたスクリーニングが必要である。	リオデジャネイロ州の大西洋岸森林地域で、これまでサルにのみ感染すると考えられていた_P.simium_が、人獣共通感染するマラリア原虫であることが明らかとなったとの報告である。

今後の対応	その他参考事項等
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、問診時にマラリアの既往歴を確認し、該当する場合は献血不適としている。さらに、リスクのあるマラリア流行地への旅行者または居住経験者の献血を一定期間延期している（1～3年の延期を行うとともに、帰国（入国）後マラリアを思わせる症状があった場合は、感染が否定されるまで、献血を見合わせる）。平成26年4月よりマラリアの献血制限対象地域を拡大し、問診を強化している。今後も引き続き、マラリア感染に関する新たな知見及び情報の収集、対応に努める。	

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation

Patrícia Brasil, Mariano Gustavo Zalis*, Anielle de Pina-Costa, Andre Machado Siqueira, Cesare Bianco Júnior, Sidnei Silva, André Luiz Lisboa Areas, Marcelo Pelajo-Machado, Denise Anete Madureira de Alvarenga, Ana Carolina Faria da Silva Santelli, Hermano Gomes Albuquerque, Pedro Cravo, Filipe Vieira Santos de Abreu, Cassio Leonel Peterka, Graziela Maria Zanini, Martha Cecilia Suárez Mutis, Alcides Pissinatti, Ricardo Lourenço-de-Oliveira, Cristiana Ferreira Alves de Brito, Maria de Fátima Ferreira-da-Cruz, Richard Culleton, Cláudio Tadeu Daniel-Ribeiro*

Summary

Background Malaria was eliminated from southern and southeastern Brazil over 50 years ago. However, an increasing number of autochthonous episodes attributed to *Plasmodium vivax* have recently been reported from the Atlantic Forest region of Rio de Janeiro state. As the *P vivax*-like non-human primate malaria parasite species *Plasmodium simium* is locally enzootic, we performed a molecular epidemiological investigation to determine whether zoonotic malaria transmission is occurring.

Methods We examined blood samples from patients presenting with signs or symptoms suggestive of malaria as well as from local howler monkeys by microscopy and PCR. Samples were included from individuals if they had a history of travel to or resided in areas within the Rio de Janeiro Atlantic Forest, but not if they had malaria prophylaxis, blood transfusion or tissue or organ transplantation, or had travelled to known malaria endemic areas in the preceding year. Additionally, we developed a molecular assay based on sequencing of the parasite mitochondrial genome to distinguish between *P vivax* and *P simium*, and applied this assay to 33 cases from outbreaks that occurred in 2015, and 2016.

Findings A total of 49 autochthonous malaria cases were reported in 2015–16. Most patients were male, with a mean age of 44 years (SD 14·6), and 82% lived in urban areas of Rio de Janeiro state and had visited the Atlantic Forest for leisure or work-related activities. 33 cases were used for mitochondrial DNA sequencing. The assay was successfully performed for 28 samples, and all were shown to be *P simium*, indicative of zoonotic transmission of this species to human beings in this region. Sequencing of the whole mitochondrial genome of three of these cases showed that *P simium* is most closely related to *P vivax* parasites from South America. The malaria outbreaks in this region were caused by *P simium*, previously considered to be a monkey-specific malaria parasite, related to but distinct from *P vivax*, and which has never conclusively been shown to infect people before.

Interpretation This unequivocal demonstration of zoonotic transmission, 50 years after the only previous report of *P simium* in people, leads to the possibility that this parasite has always infected people in this region, but that it has been consistently misdiagnosed as *P vivax* because of an absence of molecular typing techniques. Thorough screening of local non-human primates and mosquitoes (*Anopheline*) is required to evaluate the extent of this newly recognised zoonotic threat to public health and malaria elimination in Brazil.

Funding Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado de Rio de Janeiro, The Brazilian National Council for Scientific and Technological Development (CNPq), JSPS Grant-in-Aid for scientific research, Secretary for Health Surveillance of the Brazilian Ministry of Health, Global Fund, Fundaçao de amparo à pesquisa do estado de Minas Gerais (Fapemig), and PRONEX Program of the CNPq.

Introduction

Zoonotic malaria occurs when people become infected with malaria parasite species that more commonly infect non-human primates. Species such as *Plasmodium knowlesi* and *Plasmodium cynomolgi*, both parasites of macaque monkeys (*Macaca*), can infect people via the bites of infected mosquitoes under natural and experimental conditions. *P knowlesi* is responsible for a high proportion of human malaria cases in Southeast Asia, mostly affecting individuals living or working in close contact with forests.[1] Zoonotic malaria poses a unique problem for malaria control efforts and complicates the drive towards eventual elimination of the disease; because of the nature of its reservoir and transmission dynamics, the interruption of its transmission might not be achievable with the available tools in areas of high forest coverage.

Lancet Glob Health 2017; 5: e1038–46

Published Online August 31, 2017 http://dx.doi.org/10.1016/S2214-109X(17)30333-9

See Comment page e961

*Contributed equally

Laboratório de Doenças Febris Agudas (P Brasil MD, A de Pina-Costa PhD, A M Siqueira MD) **and Laboratório de Parasitologia** (S Silva MSc, G M Zanini PhD), **Instituto Nacional de Infectologia Evandro Chagas (INI), Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil; Centro de Pesquisa, Diagnóstico e Treinamento em Malária (CPD-Mal), Fiocruz, Rio de Janeiro, Brazil** (P Brasil, A de Pina-Costa, A M Siqueira, C B Júnior BSc, Prof R Lourenço-de-Oliveira PhD, Prof M de Fátima Ferreira-da-Cruz PhD, Prof C T Daniel-Ribeiro MD); **Laboratório de Infectologia e Parasitologia Molecular, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil** (M G Zalis PhD, A L L Areas MSc); **Laboratório de Pesquisa em Malária** (A de Pina-Costa, C B Júnior, Prof M de Fátima Ferreira-da-Cruz, Prof C T Daniel-Ribeiro), **Laboratório de Patologia** (Prof M Pelajo-Machado MD), **Laboratório de Doenças Parasitárias** (H G Albuquerque MSc, M C Suárez Mutis PhD), **and Laboratório de Mosquitos Transmissores de Hematozoários** (F V Santos de Abreu MSc, Prof R Lourenço-de-Oliveira), **Instituto Oswaldo Cruz (IOC),**

Fiocruz, Rio de Janeiro, Brazil; Centro Universitário Serra dos Órgãos (UNIFESO), Teresópolis, Rio de Janeiro, Brazil (A de Pina-Costa, A Pissinatti PhD)**; Laboratório de Malária, Centro de Pesquisas René Rachou por Institute René Rachou (IRR), Fiocruz, Belo Horizonte, Brazil** (D A M de Alvarenga MSc, Prof C F A de Brito PhD)**; Programa Nacional de Prevenção e Controle da Malária, Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília, Brazil** (A C F da Silva Santelli MD, C L Peterka MSc)**; Laboratório de Genoma e Biotecnologia (GenoBio), Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil** (P Cravo PhD)**; Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal** (P Cravo)**; Centro de Primatologia do Rio de Janeiro (CPRJ/INEA), Rio de Janeiro, Brazil** (A Pissinatti)**; and Malaria Unit, Department of Pathology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan** (R Culleton PhD)

Correspondence to:
Prof Cláudio Tadeu Daniel-Ribeiro, Laboratório de Pesquisa em Malária, Instituto Oswaldo Cruz (IOC-Fiocruz), Rio de Janeiro, CEP 21.040-360, Brazil
malaria@fiocruz.br
or
Dr Patrícia Brasil, Laboratório de Pesquisa em Malária, Instituto Oswaldo Cruz (IOC-Fiocruz), Rio de Janeiro, CEP 21.040-360, Brazil
patricia.brasil@ini.fiocruz.br

For more on the **cases reported by the Ministry of Health** see www.saude.gov.br/malaria

Research in context

Evidence before this study

Autochthonous malaria infections in people leaving near the Atlantic Forest in Rio de Janeiro, Brazil, were diagnosed as *Plasmodium vivax*, a human malaria parasite. The diagnosis of *P vivax* was on the basis of the morphology of the parasites as observed through microscopy of thin blood smears stained with Giemsa's solution. As malaria was thought to have been eliminated from this area over 50 years ago, it was uncertain where and when this malaria parasite pool had emerged. Cases have been increasing in the past 5 years.

Added value of this study

This study shows that these parasites are, in fact, not *P vivax*, but rather *P simium*, a closely related parasite species whose natural hosts are non-human primates native to the Atlantic Forest. This diagnosis was made by molecular investigation of parasite DNA. Genotyping of malaria parasites from monkeys in this region revealed that the same parasites are infecting both monkeys and human beings in this area.

Implications of all the available evidence

Our study suggests that malaria transmission in the Atlantic Forest region of Rio de Janeiro has a zoonotic component, with parasites shared between human beings and monkeys. The implications of this finding for malaria control and elimination in this region are profound, as zoonotic reservoirs of disease are difficult to target with interventions.

Once prevalent throughout the country, malaria transmission in Brazil now occurs almost entirely within the northern Amazon region. However, a consistent number of autochthonous cases have been reported in southern and southeastern regions of Brazil from where human malaria was eliminated 50 years ago.[2] From 2006 to 2016, 1032 autochthonous cases (Ministry of Health Brazil 2017) were reported at sites scattered along the mountainous valleys covered by the Atlantic Forest in these regions. The Atlantic Forest is rich in bromeliads (Bromeliaceae), which provide a larval habitat for *Anopheles Kertezsia cruzii*, a vector of both human and non-human primate malaria parasites. Most of the malaria cases reported in the Atlantic Forest have been attributed to *Plasmodium vivax* and mainly occur among non-resident visitors, without any identifiable index case that could have introduced the parasite from a malaria endemic region.[3]

It has long been hypothesised that autochthonous human malaria in the Atlantic Forest could be the result of infection by non-human primate parasite species.[4] In 1966, Deane and colleagues[4] proposed that monkeys could serve as reservoirs of *Plasmodium* that could be transmitted to people by *A K cruzii*, because this species is known to bite both monkeys in the forest canopy and people at ground level.

Two malaria parasite species are known to infect new world monkeys (Ceboidea) in the Atlantic Forest of Brazil: *Plasmodium simium* and *Plasmodium brasilianum*.[5] These are similar at the morphological, genetic, and immunological levels to *P vivax* and *Plasmodium malariae*, respectively.[6] *P simium* has been observed to naturally infect howler monkeys of the genera *Alouatta* and *Brachyteles*, and capuchin monkeys of the genera *Cebus* and *Sapajus*.[7] Despite the distribution of the howler monkeys and capuchins across almost all biomes in South and Central America, the distribution of *P simium* is considered to be limited to the Atlantic Forest of south and southeastern Brazil.[5]

Here we describe the parasitological and molecular analyses of parasites causing autochthonous human malaria in the Atlantic Forest region of Rio de Janeiro in 2015 and 2016, with the aim of determining whether zoonotic malaria transmission occurs there.

Methods

Study area, population, and design

Rio de Janeiro state is located in southeast Brazil. It consists of urban areas with high population densities, mostly in the coastal lowlands, and mountainous areas covered by the Atlantic Forest containing small cities and settlements scattered in the valleys. Localities where malaria cases have been reported are situated in valleys between 280 m and 1300 m above sea level.[8]

We performed an epidemiological investigation to characterise the possible location of infection, by classifying each episode as autochthonous or imported. The cases considered here are from patients who attended the Instituto Nacional de Infectologia Evandro Chagas (INI), a reference centre for the diagnosis and treatment of infectious diseases at the Fundação Oswaldo Cruz (Fiocruz), in Rio de Janeiro, Brazil. Blood samples from patients with acute fever symptoms were collected from the Acute Febrile Illness Outpatient Clinic in INI. The INI-Fiocruz Ethical Board approved the study (number 0062.0.009.000-11). All participants provided informed written consent.

Procedures

Individuals were recruited upon presentation of signs or symptoms suggestive of malaria, a history of travel to or habitation in areas within the Rio de Janeiro Atlantic Forest, and a positive test by thick blood smear or PCR, or both. Individuals were excluded if they had malaria prophylaxis, blood transfusion or tissue or organ transplantation, used intravenous drugs, had a needlestick injury, resided or undertook recreation near ports or airports, or travelled to known malaria endemic areas in the preceding year. Following informed consent, venous blood was drawn for clinical laboratory analyses and molecular studies. Additional tests, such as blood

culture, viral serology and G6PD deficiency, were done for all patients at the attending physician's discretion.

Diagnosis by microscopy

Giemsa's solution-stained thin and thick blood smears were examined by bright-field microscopy, with a 100×/1·3 numerical aperture oil immersion objective lens for species identification and parasite density estimations. Blood films were examined for a minimum of 100 fields in the case of the presence of malaria parasites and 500 fields when no parasites could be detected. Parasite numbers were recorded per 200 white blood cells, or 500 white blood cells in the case of low parasitaemia. To estimate parasite density, a standard mean white blood cell count of 6000 white blood cells per µL of blood was assumed. All slides were subsequently examined by an independent Pan American Health Organization or WHO accredited malaria microscopist.

DNA extraction and *P vivax* species-specific PCR

DNA was extracted from whole blood with the QIAamp midi kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA samples were tested for *P vivax* by conventional and real-time PCR (rtPCR), both using the cysteine proteinase gene (GenBank number L26362) as a target.[9] For rtPCR, 2·5 µL of DNA were added to a 47·5 µL mixture containing the 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 300 nM of primer Pv1 (5′ATCAACGAGCAG ATGGAGAAATATA3′), 300 nM of primer Pv5 (5′GCT CTCGAAATCTTTCTTCGA3′), and 150 nM of PVIV probe (5′ FAM AACTTCAAAATGAATTATCTC MGB NFQ 3′) (Applied Biosystems). Amplification conditions involved two holds (50°C for 2 min and 95°C for 10 min) followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). The rtPCR was run at least in duplicate on the ABI PRISM Sequence Detection System 7500 (Applied Biosystems). The results were analysed using ABI Prism 7500, software version 1.1 RQ Study. TaqMan RNaseP Control 20x was used as an endogenous reaction control. To avoid DNA contamination, we used separated workstations for mix preparation and DNA extraction and we applied DNA Away for surface decontamination. Positive (DNA extracted from blood from patients with known *P vivax* infection) and negative (no DNA and DNA extracted from individuals who have never travelled to malaria-endemic areas) controls were used in each round of amplification. This PCR does not discriminate between *P vivax* and *P simium*.

Non-human primate samples

DNA was extracted from samples obtained from four howler monkeys from different sites and times in southeastern Brazil (MB CPRJ from Guapimirim in December, 2013; RJ 30 from Vale das Princesas, Miguel Pereira on March 21, 2016; RJ 59 from Macaé on Sept 22, 2016; and ATCC from São Paulo in 1966).

DNA extracted from the spleen and liver of one brown howler monkey (*Alouatta guariba clamitans*), found dead in Guapimirim (one of the municipalities where human malaria occurs in the Atlantic Forest of Rio de Janeiro), was used for *Plasmodium* species detection by nested-PCR.[10] Samples from both organs were positive for *P vivax* or *P simium* DNA, according to our PCR method.[7]

DNA was also extracted from the blood of two *A g clamitans*; one was captured at Vale das Princesas, Miguel Pereira (a site where human malaria cases have also been reported in Rio de Janeiro) in 2016, and tested positive by PCR analysis for both *P vivax* or *P simium* and *P brasilianum* or *P malariae*, and the other was from Macaé (another locality in Rio de Janeiro with human malaria cases), and was positive for *P vivax* or *P simium*. Additionally, a *P simium* reference sample (American Type Culture Collection [ATCC] 30130), derived from a howler monkey (*Alouatta fusca clamitans*) captured in São Paulo, southeast Brazil, in 1966, was also used. The DNA extracted from these four monkey samples also underwent mitochondrial genome analysis.

For more on the **reference sample** see https://www.atcc.org/~/ps/30130.ashx

Molecular phylogenetic analysis of *P simium* infections

Among samples derived from 39 individuals presenting at INI, 33 were subjected to parasite mitochondrial genome sequencing (20 from 2015 and 13 from 2016): 30 had partial analysis and three full-length mitochondrial genome sequencing. Samples from two monkeys collected from the Atlantic Forest of Rio de Janeiro and one ATCC *P simium* reference sample were also subjected to malaria parasite mitochondrial genome sequencing.

Because of the low amount of high-quality parasite DNA, full-length mitochondrial genome sequence was obtained for only four samples (three cases: AF 1, AF 2, and AF 3 and the ATCC reference sample), following the method reported by Culleton and colleagues,[11] and was compared with 794 *P vivax* mitochondrial genome sequences and three sequences of *P simium* (accession numbers AY800110, NC_007233 and AY722798, all of which have identical sequences) deposited in Genbank.[11–17] Using these sequences, a median-joining haplotype network was produced with NETWORK 4.5.0, as previously described.[11]

The mitochondrial genome of the remaining 30 samples was partially sequenced to distinguish *P simium* from *P vivax*. *P simium* differs from the most closely related *P vivax* isolate at two unique single-nucleotide polymorphisms (SNPs) in the mitochondrial genome, at positions 3535 (T→C) and 3869 (A→G), numbered according to the nucleotide sequences deposited by Culleton and colleagues.[11] These two SNPs are close together, and can be PCR amplified and sequenced with a single set of primers, or with a nested PCR if DNA concentrations are low. Primer pairs for the outer PCR were PsimOUTF 5′CAGGTGGTG TTTTAATGTTATTATCAG3′ and PsimOUTR 5′GCATAG GTAAGAATGTTAATACAACTCC3′, whereas inner

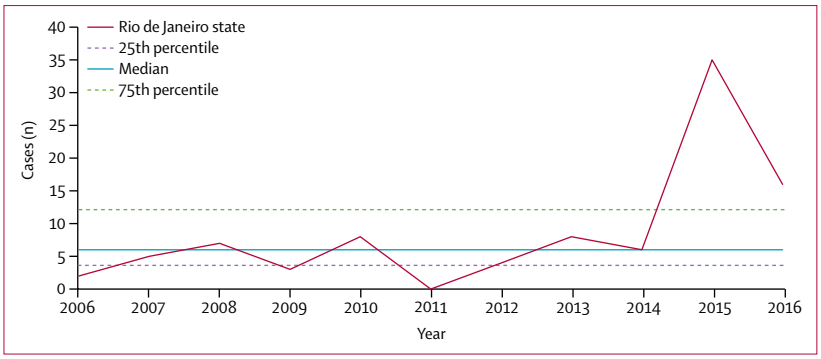


Figure 1: **Historical series of autochthonous malaria cases in the state of Rio de Janeiro, Brazil, from 2006 to 2016**
Historical series of autochthonous malaria cases from 2006 to 2016. In 2015–16, the number of cases exceeding the 75th percentile of maximum expected cases increased sharply, configuring an outbreak.

See Online for appendix

PCR primers were PsimINF 5′GCTGGAGATCCTATT TTATATCAAC3′ and PsimINR 5′ATGTAAACAATCCAA TAATTGCACC3′.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between 2006 and 2014, 43 autochthonous malaria cases were reported in the Atlantic Forest in the state of Rio de Janeiro, an average of 4·8 cases per year (SD 2·8), with an unexpected increase in the number of cases occurring during outbreaks in 2015 (n=33) and 2016 (n=16; figure 1). In 2015, 25 (76%) of the 33 cases were followed and processed at Fiocruz. In 2016 (until Oct 31), 14 of 16 (88%) cases were also investigated at Fiocruz, with a total of 39 (80%) of the 49 cases reported in the state.

Patients followed up at Fiocruz had a mean age of 44 years (SD 14·6) and median age of 50 years (range 7–82; table). Most patients were male (79%; table) and inhabitants of urban areas of Rio de Janeiro state (82%), who visited areas of the Atlantic Forest for leisure or work-related activities, spending a median of 5 days (range 1–30) in vegetation-dense areas and its close surroundings. Transmission occurred either in people who entered regions of dense vegetation coverage or in people who lived in rural areas with low-population density in mountain valleys (figure 2). The presence of monkeys was regularly reported in the neighbouring forest by the inhabitants of all areas.

Case clustering occurred only when individuals travelled together and developed symptoms in the same incubation period. Fever was the main symptom and was present in all malaria cases. Periodic tertian fever was observed in 35 cases (90%). No patient was admitted to hospital and all made full recoveries with complete cessation of symptoms following treatment. It was the first malaria episode for all patients and only one patient was G6PD deficient.

In 37 cases (95%) a diagnosis of *P vivax* was made by microscopy. The highest parasitaemia was 3000 parasites per µL of blood and, in more than 67% of the cases, it was lower than 500 parasites per µL. Two patients had negative tests for the presence of parasites by microscopy. A PCR for *P vivax*-species detection, which does not discriminate between *P vivax* and *P simium*, suggested the presence of *P vivax* in 38 patients (97%).

When compared with *P vivax* from the malaria endemic Amazonian regions, parasites from the Atlantic Forest diagnosed as *P vivax* were morphologically different (appendix). Trophozoites were pleomorphic but less amoeboid than those observed in *P vivax* (appendix). They had a large mass of chromatin and a more compact cytoplasm with malaria pigment (appendix). Usually stippling was mostly observed in infected cells with late developmental forms, but erythrocytes containing early trophozoites were also frequently stippled (figure 3A–F). Furthermore, developing schizonts contained fewer merozoites than in *P vivax* (figure 3G–L). The highest number of merozoites in mature schizonts was 14 (figure 3M). Gametocytes were round with compact cytoplasm and marked pigmentation (figure 3N–P).

Non-infected erythrocytes showed marked anisocytosis and poikilocytosis (figure 3). Poikilocytosis was represented mainly by acanthocytes, dacrocytes, and spherocytes, which occurred together on the same preparations (figure 3).

Analysis of the four usable mitochondrial genome samples from the 33 human cases used for DNA sequencing revealed that they shared identical sequences, and these were in turn identical to the mitochondrial genome sequence of *P simium* deposited at Genbank, which differs from the most closely related isolates of *P vivax* by two SNPs. Analysis of 794 full-length mitochondrial genome sequences from globally acquired *P vivax* samples showed that these SNPs were unique to *P simium*. A haplotype network tree (appendix) was constructed using these sequences, and shows that *P simium* is most closely related to the *P vivax* parasites of human beings isolated from South America.

On the basis of two informative SNPs that differentiate *P vivax* from *P simium*, we were able to diagnose an infection of *P simium* in 28 of 33 samples typed for their species (table). We were unable to achieve PCR amplification for the remaining five samples, because of technical constraints. The same informative SNPs were found in *P simium* infecting three local howler monkeys, MB CPRJ, RJ 30, and R J59 (table).

Discussion

The results of our study have important implications for public health and for the malaria elimination agenda. To our knowledge, this is the first demonstration of

P simium naturally infecting human beings in forest locations in a region considered to have eliminated transmission of malaria at least 50 years ago. The sudden increase in malaria cases in the past 2 years in that area is associated with the Atlantic Forest of Rio de Janeiro. No major environmental modifications appear to have occurred that might have modified the behaviour of *Anopheles* spp or monkeys during this

	Sample collection (year)	Age (years)	Sex	Main activity developed in the area	Visitor or resident	Entry into Atlantic Forest area	Time between onset of symptoms and diagnosis (days)	Triad of malaria*	Highest axillar temperature (°C)	Parasites density (mm³/μL)	*Plasmodium simium* SNPs†
Human samples											
AF 1	2015	34	Male	Photographer	Visitor	Yes	11	Yes	39·5	920	Yes‡
AF 2	2015	58	Male	Engineering	Visitor	No	12	Yes	38·6	560	Yes‡
AF 3	2015	50	Male	Geologist	Visitor	Yes	14	Yes	38·8	112	Yes‡
AF 4	2015	27	Male	Ecotourism	Visitor	Yes	15	Yes	38·1	1200	Yes
AF 5	2015	26	Male	Ecotourism	Visitor	Yes	14	Yes	39·0	64	Yes
AF 6	2015	51	Male	Inhabitant	Resident	Yes	14	No	38·5	480	Yes
AF 7	2015	40	Male	Ecotourism	Visitor	Yes	13	Yes	39·5	800	Yes
AF 8	2015	52	Male	Ecotourism	Visitor	Yes	10	Yes	39·8	416	Yes
AF 9	2015	29	Male	Ecotourism	Resident	Yes	6	Yes	38·0	64	Yes
AF 10	2015	35	Male	Architecture	Visitor	Yes	16	Yes	39·0	576	Yes
AF 11	2015	52	Female	Ecotourism	Visitor	Yes	12	Yes	38·8	320	Yes
AF 12	2015	48	Male	Inhabitant	Visitor	No	9	Yes	NA	208	Yes
AF 13	2015	52	Male	Forestal Garden	Resident	Yes	12	Yes	40·0	624	Yes
AF 14	2015	26	Male	Ecotourism	Visitor	Yes	13	Yes	40·0	128	Not determined§
AF 15	2015	44	Male	Ecotourism	Visitor	Yes	39	Yes	39·0	1296	Yes
AF 16	2015	59	Male	Ecotourism	Visitor	Yes	20	Yes	40·0	336	Yes
AF 17	2015	54	Male	Ecotourism	Visitor	Yes	6	Yes	39·8	96	Not determined§
AF 18	2015	39	Male	Ecotourism	Visitor	Yes	3	No	38·0	112	Yes
AF 19	2015	56	Male	Ecotourism	Visitor	Yes	16	Yes	39·0	480	Yes
AF 20	2015	22	Male	Engineering	Visitor	No	NA	No	39·5	80	Not determined§
AF 21	2016	82	Female	Tourism	Visitor	No	13	Yes	NA	3000	Yes
AF 22	2016	40	Male	Ecotourism	Resident	Yes	10	Yes	39·0	48	Yes
AF 23	2016	35	Female	Inhabitant	Resident	No	12	Yes	39·0	Negative	Yes
AF 24	2016	50	Male	Ecotourism	Visitor	Yes	14	Yes	NA	80	Yes
AF 25	2016	26	Male	Ecotourism	Visitor	Yes	9	Yes	38·5	672	Yes
AF 26	2016	55	Male	Ecotourism	Visitor	Yes	14	No	39·0	1160	Yes
AF 27	2016	54	Female	Ecotourism	Visitor	Yes	18	Yes	40·0	2600	Yes
AF 28	2016	54	Male	Ecotourism	Visitor	Yes	3	No	38·5	592	Yes
AF 29	2016	51	Female	Ecotourism	Resident	Yes	11	Yes	38·0	416	Not determined§
AF 30	2016	52	Male	Ecotourism	Visitor	Yes	16	Yes	41·9	384	Yes
AF 31	2016	54	Female	Ecotourism	Visitor	Yes	10	Yes	NA	80	Yes
AF 32	2016	72	Female	Ecotourism	Visitor	Yes	13	Yes	39·0	704	Yes
AF 33	2016	53	Male	Ecotourism	Visitor	Yes	NA	Yes	NA	144	Not determined§
AF 34	2015	39	Female	Ecotourism	Visitor	Yes	13	No	NA	48	Not done
AF 35	2015	7	Male	Tourism	Visitor	No	12	Yes	39·7	48	Not done
AF 36	2015	47	Male	Ecotourism	Visitor	Yes	18	No	38·5	122	Not done
AF 37	2015	53	Male	Ecotourism	Visitor	Yes	21	Yes	39·7	80	Not done
AF 38	2015	22	Male	Gardener	Resident	Yes	9	Yes	NA	Negative	Not done
AF 39	2016	42	Male	Ecotourism	Visitor	Yes	20	Yes	39·0	256	Not done

(Table continues on next page)

	Sample collection (year)	Age (years)	Sex	Main activity developed in the area	Visitor or resident	Entry into Atlantic Forest area	Time between onset of symptoms and diagnosis (days)	Triad of malaria*	Highest axillar temperature (°C)	Parasites density (mm³/μL)	*Plasmodium simium* SNPs†
(Table continued from previous page)											
Non-human primates											
ATCC 30130	1966	..	..	..	..	..	..	..	..	..	Yes‡
MB CPRJ	2013	..	..	..	..	..	..	..	..	..	Yes
RJ 30	2016	..	..	..	..	..	..	..	..	..	Yes
RJ 59	2016	..	..	..	..	..	..	..	..	..	Yes

SNP=single-nucleotide polymorphisms. NA=not available. ATCC=American Type Culture Collection. *Fever, chills or rigors, and sweating. †SNPs identified by partial mitochondrial genome sequencing. ‡SNPs identified by whole mitochondrial genome sequencing. §Unable to achieve PCR amplification because of technical constraints.

Table: **Clinical, epidemiological, and parasitological characteristics of studied samples and identification of *Plasmodium simium* SNPs through whole or partial mitochondrial genome sequencing**

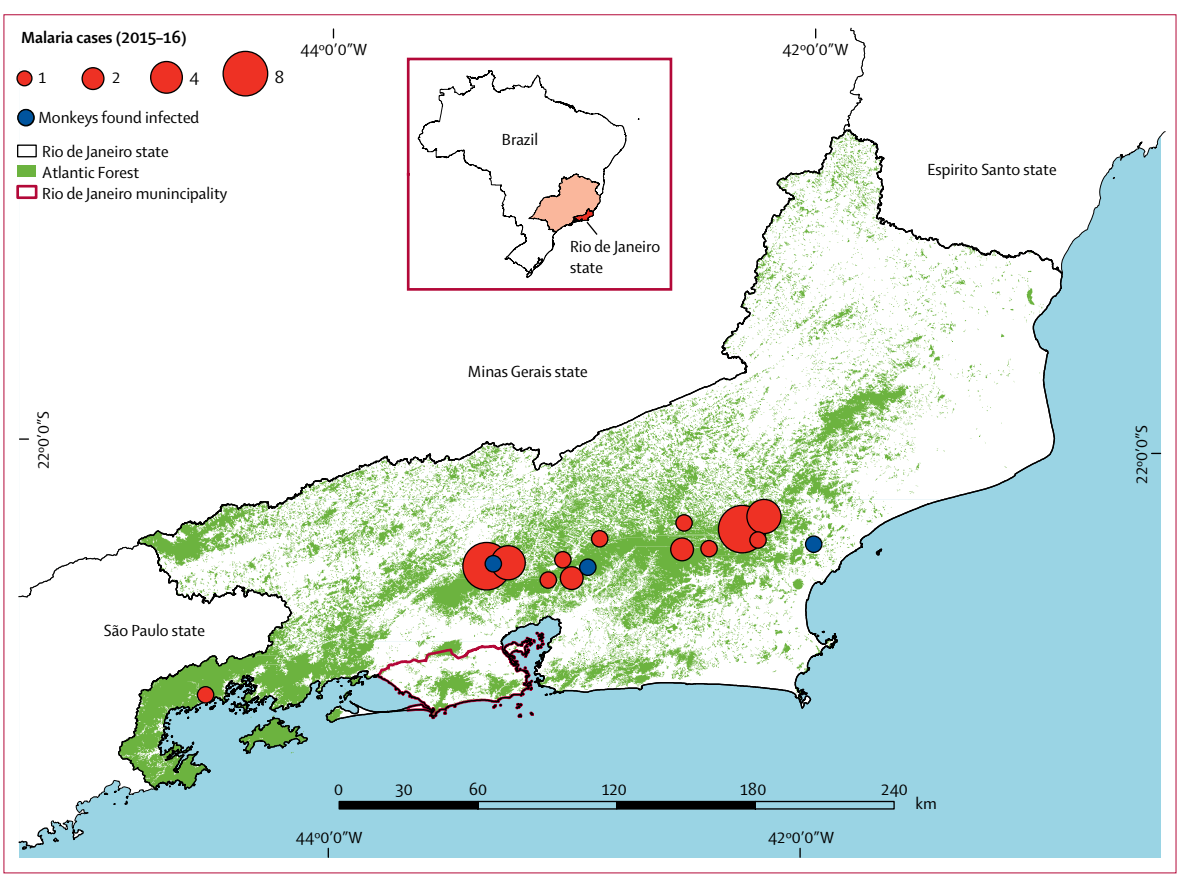


Figure 2: **Map of the Rio de Janeiro state, Brazil, showing the Atlantic Forest and indicating where human malaria cases of simian origin and monkeys infected with *Plasmodium simium* have been detected**

Human cases are represented by red spots of different sizes (symbolising one to eight cases), and the three captured, infected, wild howler monkeys are shown as blue spots. The extension of the area covered by the Atlantic Forest vegetation is indicated in green. All cases were reported in forest fragments located in Serra do Mar, and monkeys carrying *P simium* were found in the vicinity of each area. The municipality of Rio de Janeiro, delimitated with the red bold line, is free of malaria transmission.

time. However, the recent rise of ecotourism and the so-called back to nature movement might increase the opportunities for vector sharing between monkeys and human beings in this region. Despite increasing urbanisation, most of Brazil remains forested, with many human populations living in close contact with forests. The 2017 outbreak of sylvatic yellow fever in southeastern Brazil, a well established zoonosis that

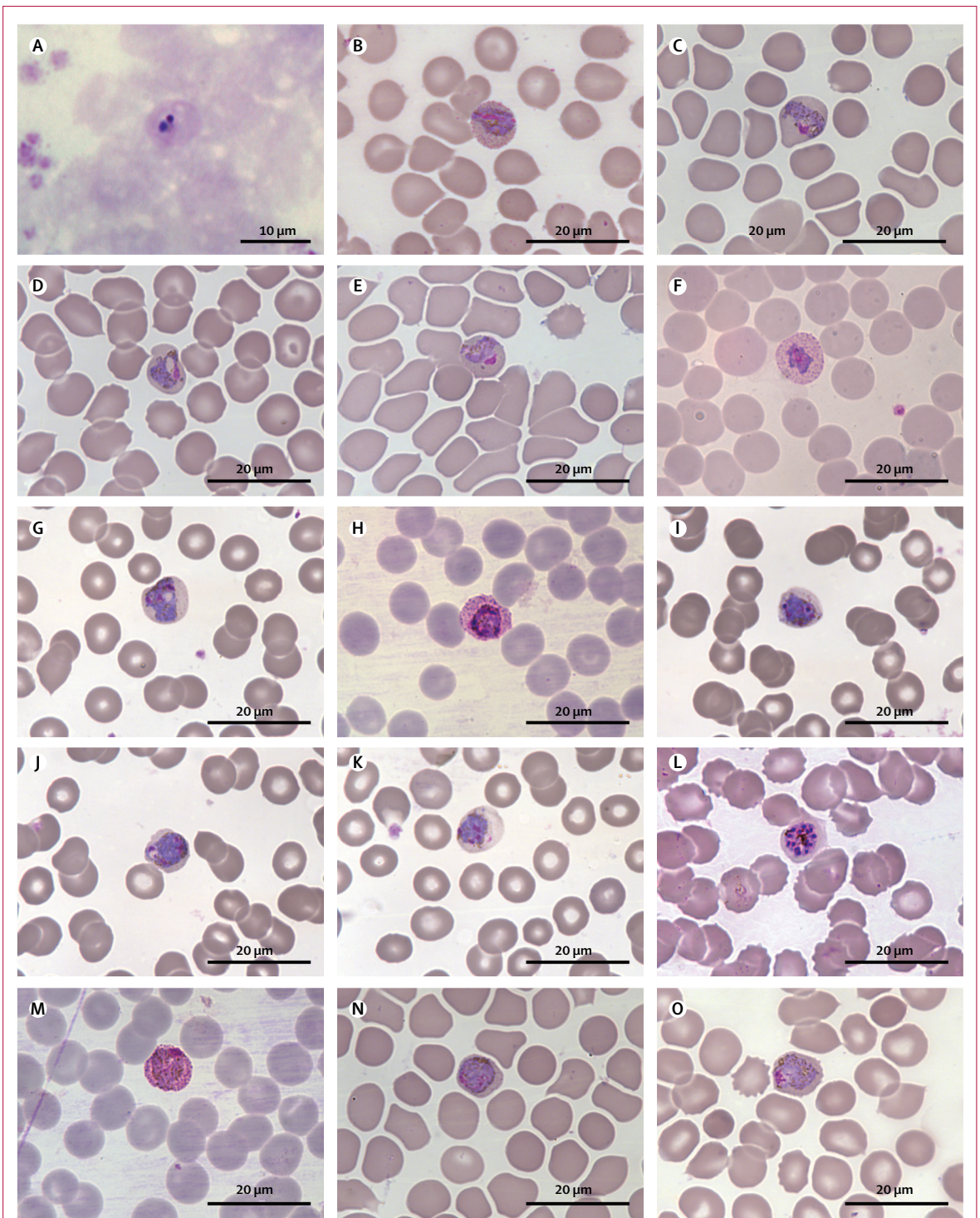


Figure 3: **Giemsa's solution-stained preparations of blood samples from human beings naturally infected with *Plasmodium simium* in Rio de Janeiro state, Brazil**
All preparations are thin blood films, except A (thick blood smear). (A) Early trophozoite. (B–F) Pleomorphic developing trophozoites. (G–L) Immature schizonts. (M) Mature schizont. (N–P) Gametocytes.

affected at least five Brazilian states, should raise concern for the possibility of the extension of occurrence of zoonotic malaria, because of the resemblance of the environmental and demographic characteristics in which both infections occur.[18] Further research is needed to elucidate these aspects.

P simium, a tertian malaria parasite found in New World non-human primates was first identified in 1951 in a monkey from the state of São Paulo and appears to be restricted to the Atlantic Forest regions of southern and southeastern Brazil.[5,19,20] Fonseca (1951),[21] Garnham (1966),[22] and Deane and colleagues (1966)[4] highlighted the morphological differences between *P vivax* and *P simium*; the trophozoites of *P simium* being less amoeboid and with coarser and more precocious and very prominent Schüffner's dots than *P vivax*. Garnham (1966)[22] reported that the detection of stippling in *P simium* early parasitised cells depends on the staining procedures. These morphological characteristics of *P simium* are consistent with those described here for the infections of human beings from the Atlantic Forest.

Although the initial diagnoses for these infections was *P vivax*, molecular evidence has revealed that these parasites are *P simium*. This misdiagnosis of a zoonotic non-human primate malaria parasite as a human parasite species has precedent and parallels the discovery of the large focus of *P knowlesi* in Borneo, which was initially attributed to *P malariae* on the basis of morphological characteristics.[23]

Despite the apparent genetic similarity of *P simium* to *P vivax*, attempts at inducing infections of *P simium* in human beings under laboratory conditions have been unsuccessful.[24] In 1966, however, Deane and colleagues[4] described the infection of a man with a *P vivax*-like parasite that they considered to be *P simium* on the basis of morphological characteristics of the parasite and because infection had occurred in a forest reserve outside São Paulo, where *P simium* was known to be transmitted. This infection remains the only previous case report of a possible human infection with *P simium*.

The clinical and parasitological features of our cases reveal that the pyrogenic threshold of *P simium* infection is considerably low. Whether this low fever threshold is related to the naive status of the individuals or specific parasitic-associated characteristics (eg, GC-content and other inflammatory factors) are yet to be better investigated.[25,26]

Patients who were naturally infected with *P simium* reported clinical symptoms congruent to symptoms of *P vivax* malaria, and responded successfully to chloroquine and primaquine, with no hospital admission, relapses, or deaths. It is not known whether *P simium* is capable of producing hypnozoites in human beings and, thus, relapses, as does *P vivax*. However, one patient (AF 3) who was treated solely with chloroquine because of G6PD deficiency and one other patient (AF 21) who discontinued primaquine treatment due to adverse events did not present any symptomatic relapse and were always negative for *Plasmodium* in all parasitological and molecular tests done during 18 months' follow-up. Further studies will be required to establish if *P simium* is capable of producing hypnozoites.

Whether this parasite can be transmitted from person to person is not known. All patients who presented with disease had entered the forest or visited the forest surroundings inhabited by howler monkeys, the main host of *P simium*. Case clustering occurred only when patients had entered such regions together, and in these cases the same time to onset of disease symptoms was observed. Although gametocytes were detected in blood smears of *P simium*-infected individuals in the present study, the infectivity of human infections of *P simium* mosquitoes is yet to be determined. Vector competence of primatophilic mosquitoes other than *A K cruzii* for *P simium* has not been studied and is a subject that needs to be urgently addressed.

Thorough screening of a large number of the local non-human primate and mosquito (*Anopheline*) populations in this area is required to evaluate the extent of this newly recognised zoonotic threat to public health. Moreover, one limitation of this study is the inclusion of samples from only one state covered by the Atlantic Forest. The analysis of both human and non-human primate samples from other areas that have been collected at different times will clarify whether the SNPs used to distinguish *P vivax* from *P simium* are specific to this region and this specific timeframe. However, the ATCC monkey sample was collected in a different region and time (50 years before) and it contains the same *P simium*-specific SNPs observed in the Rio de Janeiro Atlantic Forest. The small number of sequences from *P simium* hampers further analysis, and precludes drawing any conclusions regarding the evolution, natural history, and species status of this parasite.

This unequivocal demonstration of zoonotic *P simium* transmission leads to the possibility that this parasite, consistently misdiagnosed as *P vivax* because of an absence of molecular typing techniques, has always infected human beings in this region. Alternatively, it might be the case that *P simium* has only recently acquired the ability to frequently infect human beings, and this scenario has extremely important implications in terms of parasite–host relationships and evolution.

In summary, we report that the malaria outbreaks in 2015 and 2016 in the Atlantic Forest of southeastern Brazil were caused by *P simium*, previously considered to be a monkey-specific species of malaria parasite that is related to but distinct from *P vivax*, and which has never conclusively been shown to infect human beings before. Such zoonotic transmission of a malaria parasite from a monkey reservoir to human beings has immediate consequences for public health in this region, and for future attempts to control and eventually eliminate malaria in Brazil. Thorough screening of the local non-human primate and mosquito (*Anopheline*) populations in this area is required to evaluate the extent of this newly recognised zoonotic threat to public health.

Contributors

PB and CTD-R conceived the study. PB and AMS clinically followed-up the patients and AdP-C and CBJ obtained patients' data. FVSdA, RL-d-O, DAMdA, CBJ, AdP-C, and AP worked with the non-human samples. ACFdSS and CLP provided data from the National Program of Malaria Control from the Brazilian Ministry of Health. SS and GMZ examined (and RL-d-O reviewed) the microscopic slides and analysed the parasitological data. RL-d-O and SS contributed to the description of parasite morphological characteristics and SS did the slide photographs. MP-M described the red blood cell morphological characteristics. DAMdA and CFAdB undertook the molecular diagnosis of non-human primate samples. MdFF-d-C undertook the molecular diagnosis of human samples. ALLA carried out the mitochondrial genome sequence. MGZ, DAMdA, CFAdB, PC, MdFF-d-C, and RC did the analysis and interpretation of molecular data, and MGZ and RC did the DNA sequence analysis and the haplotype network in human and non-human primate samples. FVSdA and RL-d-O captured, made parasitological analysis, and interpreted non-human primate data. HGA and MCSM did the geographical description of the Atlantic Forest sites and the maps. PB, AdP-C, AMS, CFAdB, RL-d-O, RC, and CTD-R drafted and finalised the manuscript. All authors read, made suggestions, and approved the final manuscript.

Declaration of interests

We declare no competing interests.

Acknowledgments

We would like to thank Ana Paula Barroso Teixeira de Freitas and Ana Cláudia Ribeiro Fiuza for their assistance on the parasitological diagnosis, Aline Lavigne for undertaking the PCR for *P vivax*, and Igor José Silva for help with the photography of the slides. We are also grateful to Silvia Bahadian Moreira, for the facilities provided at the Primate Centre of Rio de Janeiro, and to Cristina Maria Giordano Dias, Head for the Management of Vector Transmitted Diseases and Zoonosis of the State of Rio de Janeiro (Coordination of Epidemiological Surveillance, Superintendence of Epidemiological and Environmental Surveillance) and her team for their data for patients with malaria examined outside Fiocruz. AdP-C is supported by a postdoctoral fellowship from the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado de Rio de Janeiro. The Brazilian National Council for Scientific and Technological Development (CNPq) supports PC, RL-d-O, CFAdB, MdFF-d-C and CTD-R through a Research Productivity Fellowship. RL-d-O, MdFF-d-C and CTD-R are "Cientistas do nosso Estado" from the Faperj. RC was supported by a JSPS Grant-in-Aid for scientific research grant number 16K21233. The study received financial support from the Secretary for Health Surveillance of the Ministry of Health (Brazil), through the Global Fund (agreement IOC-005-Fio-13), Fundaçao de amparo à pesquisa do estado de Minas Gerais (Fapemig), and from the PRONEX Program of the CNPq.

References

1 Ahmed MA, Cox-Singh J. *Plasmodium knowlesi*—an emerging pathogen. *Isbt Science Series* 2015; **10** (suppl 1)**:** 134–40.

2 Siqueira AM, Mesones-Lapouble O, Marchesini P, et al. *Plasmodium vivax* Landscape in Brazil: scenario and challenges. *Am J Trop Med Hyg* 2016; **95** (6 suppl)**:** 87–96.

3 de Pina-Costa A, Brasil P, Di Santi SM, et al. Malaria in Brazil: what happens outside the Amazonian endemic region. *Mem Inst Oswaldo Cruz* 2014; **109:** 618–33.

4 Deane LM, Deane MP, Ferreira Neto J. Studies on transmission of simian malaria and on the natural infection of man with *Plasmodium simium* in Brazil. *Bull World Health Organ* 1966; **35:** 805–08.

5 Deane LM. Simian malaria in Brazil. *Mem Inst Oswaldo Cruz* 1992; **87:** 1–20.

6 Coatney GR, Collins WE, Warren M, Contacos PG. The primate malarias. Bethesda, MD: US Department of Health, Education, and Welfare, 1971.

7 Alvarenga DA, de Pina-Costa A, de Sousa TN, et al. Simian malaria in the Brazilian Atlantic forest: first description of natural infection of capuchin monkeys (Cebinae subfamily) by *Plasmodium simium*. *Malar J* 2015; **14:** 81.

8 Brasil P, Costa AP, Longo CL, Silva S, Ferreira-da-Cruz MF, Daniel-Ribeiro CT. Malaria, a difficult diagnosis in a febrile patient with sub-microscopic parasitaemia and polyclonal lymphocyte activation outside the endemic region, in Brazil. *Malar J* 2013; **12:** 402.

9 Torres KL, Figueiredo DV, Zalis MG, Daniel-Ribeiro CT, Alecrim W, Ferreira-da-Cruz MF. Standardization of a very specific and sensitive single PCR for detection of *Plasmodium vivax* in low parasitized individuals and its usefulness for screening blood donors. *Parasitol Res* 2006; **98:** 519–24.

10 Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* 1993; **58:** 283–92.

11 Culleton R, Coban C, Zeyrek FY, et al. The origins of African *Plasmodium vivax*; insights from mitochondrial genome sequencing. *PLoS One* 2011; **6:** e29137.

12 Jongwutiwes S, Putaporntip C, Iwasaki T, Ferreira MU, Kanbara H, Hughes AL. Mitochondrial genome sequences support ancient population expansion in *Plasmodium vivax*. *Mol Biol Evol* 2005; **22:** 1733–39.

13 Mu J, Joy DA, Duan J, et al. Host switch leads to emergence of *Plasmodium vivax* malaria in humans. *Mol Biol Evol* 2005; **22:** 1686–93.

14 Iwagami M, Hwang SY, Fukumoto M, et al. Geographical origin of *Plasmodium vivax* in the Republic of Korea: haplotype network analysis based on the parasite's mitochondrial genome. *Malar J* 2010; **9:** 184.

15 Miao M, Yang Z, Patch H, Huang Y, Escalante AA, Cui L. *Plasmodium vivax* populations revisited: mitochondrial genomes of temperate strains in Asia suggest ancient population expansion. *BMC Evo Biol* 2012; **12:** 22.

16 Taylor JE, Pacheco MA, Bacon DJ, et al. The evolutionary history of *Plasmodium vivax* as inferred from mitochondrial genomes: parasite genetic diversity in the Americas. *Mol Biol Evol* 2013; **30:** 2050–64.

17 Rodrigues PT, Alves JM, Santamaria AM, et al. Using mitochondrial genome sequences to track the origin of imported *Plasmodium vivax* infections diagnosed in the United States. *Am J Trop Med Hyg* 2014; **90:** 1102–08.

18 Bonaldo MC, Gómez MM, Dos Santos AA, et al. Genome analysis of yellow fever virus of the ongoing outbreak in Brazil reveals polymorphisms. *Mem Inst Oswaldo Cruz* 2017; **112:** 447–51.

19 Wanderley DM, da Silva RA, de Andrade JC. Epidemiological aspects of malaria in the State of São Paulo, Brazil, 1983 to 1992. *Rev Saude Publica* 1994; **28:** 192–97.

20 Cerutti-Junior C, Boulos M, Coutinho AF, et al. Epidemiologic aspects of the malaria transmission cycle in an area of very low incidence in Brazil. *Malar J* 2007; **6:** 33.

21 Fonseca F. Plasmódio de primata do Brasil. *Mem Inst Oswaldo Cruz* 1951; **49:** 543–53 (in Portuguese).

22 Garnham PCC. Malaria parasites and other haemosporidia. Blackwell Scientific Publication, Oxford, 1966: 1114.

23 Singh B, Kim Sung L, Matusop A, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 2004; **363:** 1017–24.

24 Coatney GR. The simian malarias: zoonoses, anthroponoses, or both? *Am J Trop Med Hyg* 1971; **20:** 795–803.

25 Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivax malaria. *Trends Parasitol* 2009; **25:** 220–27.

26 Karunaweera ND, Ferreira MU, Hartl DL, Wirth DF. Fourteen polymorphic microsatellite DNA markers for the human malaria parasite *Plasmodium vivax*. *Mol Ecol Notes* 2007; **7:** 172–75.

感染症研究報告　調査報告書

調査報告書番号	KK-kaketsuken-2017091501	文献ID	28591404
研究報告の題目	Sexual transmission of American trypanosomiasis in humans : a new potential pandemic route for Chagas parasites		

研究報告の概要	報告企業の意見
問題点：ヒトにおけるアメリカ・トリパノソーマ症の性的伝播– シャーガス寄生虫の潜在的な新しい感染経路 ラテンアメリカのTrypanosoma cruzi（T.cruzi）感染は現在、4大陸の様々な国に広がっている。著者らは、T.cruziの性感染の可能性を検証した。 対象集団は4家族109人。そのうち21人は急性シャーガス病と診断されていた。 血液中の単核球および血清を、各被験者から、1年に1回、3年採取した。 ELISAおよび間接蛍光抗体法を用いてT.cruzi抗体を、PCRでDNAを検出した。 PCRでT.cruzi感染が76%（83/109）に認められた。一方、抗体検査では28.4%（31/109）にとどまった。 精液の検査では、82.6%（19/23）がPCRで陽性となった。陽性精液をマウスの腹腔内又は膣内に投与したところ、感染が成立した。PCRと抗体検査の結果が一致しなかったのは、妊娠初期の感染による免疫不応答（トレランス）が成立している可能性が考えられた。 以上より、T.cruziは女性又は男性からパートナーへ、性行為を介して感染する可能性が示された。	アメリカ・トリパノソーマ症は、Trypanosoma cruzi（T.cruzi）原虫に汚染された吸血昆虫のサシガメの排泄物を介して伝播する人獣共通感染症として知られているが、感染者からの血液の輸血、妊娠中や出産時の母親から新生児への母子感染、感染ドナーからの臓器移植などでも伝播するとされている。T.cruziは血流中では長さ20μmほどのC字型をした紡錘形である。虫体は網内系細胞や各種組織細胞内に侵入すると鞭毛や波動膜は消失し、直径1.5–4μmの球形あるいは卵形となり、宿主細胞が破壊されるまでこの型で二分裂によって増殖する。宿主細胞の破壊によって遊出した虫体は鞭毛型となり、再び各種細胞に侵入して増殖を繰り返す。 今回、流行地であるラテンアメリカにおける研究で、T cruziが性行為を介して感染する可能性が示唆された。感染患者の精液からPCRでT.cruziが検出され、当該精液からマウスへの感染が成立していること、家族内で妊娠初期の感染による免疫不応答が成立している可能性があることが要因として挙げられている。

今後の対応	その他参考事項等
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。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。	

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

platelets demonstrate a low rate of acute adverse transfusions reactions and reveal a safety profile similar to conventional platelet components.

Clinical Oral Abstract Session: Transfusion Transmitted Diseases -- Bacteria and Parasites

C90-A04C

Bacterial Screening of Apheresis Platelets with a Rapid Test- an 8 Year Single Center Experience

Nancy M. Dunbar* and Zbigniew M. Szczepiorkowski. *Dartmouth-Hitchcock Medical Center*

Background/Case Studies: The residual risk of bacterial contamination of single-donor apheresis platelets (AP) was recently addressed by the March 2016 FDA draft guidance to enhance the safety of platelet transfusion. This document also describes an existing pathway for AP outdate extension from 5 to 7 days using an FDA cleared rapid test (RT). Our hospital based transfusion service has used this RT to enhance the safety of AP transfusion since July 2008 and to routinely extend AP outdate to day 7 since February 2016. This study reports a 103 month experience of secondary screening of AP using a RT.
Study Design/Methods: All AP were obtained from our hospital-based donor center or one of four external suppliers. AP were screened by culture based methods post-collection and prior to entry into our inventory. From July 2008-January 2016, AP underwent RT on day 4. Day 6 and 7 units were transfused with physician approval when deemed medically necessary. Any units remaining in inventory on Day 8 had a second RT performed. From February 2016-January 2017, AP underwent RT on day 5 with routine outdate extension to 7 days by performing a second RT on day 6 and a third RT on day 7, as per manufacturer instructions. Any positive RTs were repeated in triplicate. Repeat RT positive units were quarantined and cultured to identify true positives. False positives (FP) were defined as repeat RT negative (type 1) or repeat RT positive with negative confirmatory culture (type 2). All RT results were reviewed during both study periods. AP transfusion and outdate rates were also summarized.
Results/Findings: Since July 2008, 20,010 AP were entered into inventory. Of these, 11,840 (59%) were transfused prior to RT testing. The remaining 8170 (41%) underwent RT on day 4 or day 5. Of these 43 (0.5%) were RT positive (29 type 1 FP, returned to inventory; 14 type 2 FP, discarded), leaving a total available inventory of 8156 units tested by RT. Of these, 5631 (28% of original inventory) were transfused before the end of day 5 and the remaining 2525 (13% of original inventory) reached a day 5 outdate. A total of 1561 (8% of original inventory) were transfused on day 6 or day 7. Of these, 768 underwent a second RT on day 6 (2 RT positives; 1 FP type one and 1 FP type 2) and 233 underwent a third RT on day 7 (no positive results). A total of 964 (5% of original inventory) outdated on day 7. Of these, 754 underwent a second RT on day 8 (no positive results).
Conclusion: To date we have performed 9925 RTs on AP at our hospital. No true positives have been identified. Use of RT over the study period decreased our outdate rate from a predicted 13% to only 5%. A total of 1522 AP have been tested twice by RT (768 on day 5 and 6; 754 on day 4 and 8) with 2 (0.1%) positive results, both of which were deemed FP by repeat testing or culture. A total of 233 units have been tested 3 times (day 5, day 6 and day 7) with no additional positives identified. We have not yet identified any units with an initial negative RT result that subsequently converted to a true positive. There is a low FP rate which should also be expected when performing repeat testing on the same unit. These data suggest that the yield for repeating the RT every 24 hours, as currently specified by the manufacturer instructions, is quite low. Additional studies are needed to clarify how RT can optimally be used to enhance detection of AP bacterial contamination.

C91-A04C

Survival of *Trypanosoma Cruzi* in Human Blood Components

Laura Tonnetti*, Aaron Thorp and Susan L Stramer. *American Red Cross*

Background/Case Studies: *Trypanosoma cruzi,* the agent of Chagas disease, is associated with 8 to 10 million infections worldwide, mostly in Latin America. Despite the extensive immigration from endemic areas, only 5 cases of transfusion-transmission (TT) *T. cruzi* have been reported in the US, before blood donor screening was implemented in 2007. Contributing factors to the low number of TT cases are a possible association between parasite lineage and TT, and high numbers of unreported cases. Platelets are almost exclusively involved in *T. cruzi* TT cases; however, during preparation of components a large fraction of the parasites can be found in red blood cells (RBCs). We investigated if blood component preparation and storage time affect the survival of the parasite and thus play a role in TT of *T. cruzi*.
Study Design/Method: Whole blood (WB) units were spiked with *T. cruzi* trypomastigotes to a final concentration between 10-10,000 parasites/mL. Each parasite concentration in WB was tested x2. An aliquot of contaminated WB was used to prepare hemocultures to detect live parasites before preparation of components. RBCs were separated and half of the components leukoreduced (LR) by filtration. Platelets and plasma were separated, along with one aliquot of plasma collected before LR. RBCs were stored at 4°C for up to 42 days; platelets were stored at 22°C (RT) under agitation for 5 days and plasma was frozen at -20°C. Aliquots for culture were removed weekly from RBCs, daily from platelets and after 30 days from frozen plasma. All samples were cultured in Liver Infusion Tryptose (LIT) media at 27°C for detection of live parasites for up to 16 weeks.
Results/Finding: Hemocultures from spiked-WB were positive at all concentration of parasites. LR'd and non-LR'd RBCs cultured before storage were positive at all concentrations. After storage at 4°C, RBCs from all units spiked with 10,000 parasites/mL were positive for up to 21 days; all further times yielded negative results. At lower concentrations, only non-LR'd RBCs spiked with 1000 parasites/mL were positive for up to 7 days. Plasma samples cultured before freezing were positive at the highest concentration in one non-LR'd sample, while all others were negative. Platelets obtained from WB spiked with 10,000 and 1000 parasites/mL were positive up to 5 days at RT. No parasites were observed in plasma or platelets prior to storage at lower concentrations. Molecular analysis to determine the presence of parasite DNA in each component is on-going.
Conclusion: Platelet storage conditions offer a suitable environment for *T. cruzi* survival; however, high concentrations of parasites also survived in RBCs at 4°C for up to 3 weeks. Leukoreduction offers partial protection, while freezing conditions appears unsuitable for *T. cruzi* survival.

C92-A04C

Hemovigilance Monitoring of Platelet Septic Transfusion Reactions (STR) after Treatment with INTERCEPT™ Pathogen Reduction or Large Volume, Delayed Bact/ALERT™ Bacterial Culture Screening

Richard Benjamin*[1], Marion Lanteri[2] and Larry Corash[1]. [1]*Cerus Corporation,* [2]*Scientific Affairs Department, Cerus Corporation*

Background/Case Studies: Amotosalen/ultraviolet A (UVA) light (INTERCEPT™ Blood System, Cerus Corporation) pathogen reduction (PR) and delayed, large volume, bacterial culture with the BacT/ALERT™ System (DLVBC) (BioMerieux, Inc) represent respective best-in-class systems to reduce the risk of STR associated with platelet concentrates (PC). Where implemented, hemoviligance (HV) programs continue to receive reports of suspected STR, most of which have low imputability as other causes are more likely or insufficient information is available to impute system failure.
Study Design/Methods: United Kingdom (2006-2015), French (2006 - 2015), Swiss (2011 - 2015), and Belgium(2009 -2015) HV reports, and Cerus Corporation's adverse event records were reviewed to assess the residual risk and imputability of STR with amotosalen/UVA-treated or DLVBC-screened PC.
Results/Findings: Approximately 1.35 million DLVBC-screened were issued with a 7 day outdate after release into inventory 3 days after collection, and ~2.3 million amotosalen/UVA-treated PC were released into inventory on day 1 or 2, with a 5 to 7 day shelf-life. No septic fatalities were reported with either technology. The French, Belgium and Swiss HV programs monitored >2.83 million conventional, non-DLVBC-screened PC and recorded 58 STR and 9 fatalities. Concurrently, zero definite and 2 possible STR were reported with 607,871 amotosalen/UVA-treated PC, significantly fewer than with conventional PC (Table 1) **(20.5 STR per million vs. 0.0 per million, P<0.001).** One definite, 1 possible, 7 undetermined/indeterminate non-fatal STR and 5 contaminated "near miss" PC were reported with 1.35 million DLVBC-screened PC between 2010 and 2015, for a reduced false-negative rate compared with the prior five years **(3.7 STR per million vs. 16.3 per million, P <0.05).** HV programs highlight a major weakness when reporting STR. Stringent criteria are used to determine definite imputability, including evidence of patient infection, PC contamination and irrefutable evidence of a donor source, with confirmation of strain identity. Reports with incomplete investigations are considered undetermined or indeterminate, or possible sepsis. Some of these cases are almost certainly due to bacterial contamination of PC, suggesting that the actual rates of sepsis are considerably higher than that reported by HV programs.
Conclusion: Best-in-class pathogen reduction and bacterial culture systems reduce STR risk, although underreporting and inadequate clinical data may result in underestimation of the true rates. Pathogen reduction of

感染症研究報告　調査報告書

調査報告書番号	KK-JBPO-K20180002	文献ID	28003547
研究報告の題目	Detection of prions in the plasma of presymptomatic and symptomatic patients with variant Creutzfeldt-Jakob disease		

研究報告の概要

vCJD発症前及び症候性患者の血漿中プリオンの検出：

変異型クロイツフェルト・ヤコブ病（vCJD）は、牛海綿状脳症（BSE）を引き起こす病原体によって汚染された肉製品の消費に起因するヒトプリオン病である。輸血により病気を伝播し得る潜在的キャリアの存在を支持する証拠が増えている。症候性vCJD患者と発症前キャリア両方の血液スクリーニングアッセイの開発は急務である。我々はフランスとイギリスのvCJD症例からの微量の異常プリオンタンパクをフェムトモルレベルで検出する、プラスミノゲンビーズキャプチャーとPMCA技術を組み合わせたアッセイを提示する。このアッセイは、最も多く罹患している2つの国のブラインドされた256の血漿サンプルから18人のvCJD患者の同定を可能とした。感度は100%（95%CI:81.5-100%）、分析特異度は99.2%（95%CI:95.9-100%）、診断特異度は100%（95%CI:96.5-100%）であった。このアッセイはまた、後にvCJDを発症した2人の献血者における臨床的発症前1.3－2.6年前のプリオンの潜在的感染の同定を可能とした。これらのデータは血液を用いたvCJD診断テストとして、PMCA技術のバリデーションに向けた重要なステップであり、輸血によるvCJD伝播リスクを制限するために必要となる発症前診断の可能性を示す。

報告企業の意見

血漿分画製剤は理論的なvCJD伝播リスクを完全には排除できないため、投与の際には患者への説明が必要である旨を2003年5月から添付文書に記載している。2009年2月17日、英国健康保護庁(HPA)はvCJDに感染した供血者の血漿が含まれる原料から製造された第Ⅷ因子製剤の投与経験のある血友病患者一名から、vCJD異常プリオン蛋白が検出されたと発表したが、弊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、国際獣疫事務局（OIE）により、日本及び米国は「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、プリオン病とされるアルツハイマー病とパーキンソン病については輸血を介して感染しないことが示唆されている。CJDは種類によって感染性が異なることが知られており、脳・脊髄、網膜などに異常型プリオン蛋白質が蓄積されるsCJDは、vCJDとは異なり、血液を介した感染を示す報告はこれまでのところないが、その潜在的可能性を示唆する報告もあるため、今後もCJDの情報については注意深く情報の収集、監視に努める。

今後の対応

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。

その他参考事項等

代表として献血ヴェノグロブリンIH5%静注0.5g/10mL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。

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

PRION DISEASES

Detection of prions in the plasma of presymptomatic and symptomatic patients with variant Creutzfeldt-Jakob disease

Daisy Bougard,[1]* Jean-Philippe Brandel,[2,3,4] Maxime Bélondrade,[1] Vincent Béringue,[5] Christiane Segarra,[1] Hervé Fleury,[6] Jean-Louis Laplanche,[4,7] Charly Mayran,[1] Simon Nicot,[1] Alison Green,[8] Arlette Welaratne,[3] David Narbey,[9] Chantal Fournier-Wirth,[1] Richard Knight,[8] Robert Will,[8] Pierre Tiberghien,[9,10] Stéphane Haïk,[2,3,4] Joliette Coste[1,9]*

Variant Creutzfeldt-Jakob disease (vCJD) is a human prion disease resulting from the consumption of meat products contaminated by the agent causing bovine spongiform encephalopathy. Evidence supporting the presence of a population of silent carriers that can potentially transmit the disease through blood transfusion is increasing. The development of a blood-screening assay for both symptomatic vCJD patients and asymptomatic carriers is urgently required. We show that a diagnostic assay combining plasminogen-bead capture and protein misfolding cyclic amplification (PMCA) technologies consistently detected minute amounts of abnormal prion protein from French and British vCJD cases in the required femtomolar range. This assay allowed the blinded identification of 18 patients with clinical vCJD among 256 plasma samples from the two most affected countries, with 100% sensitivity [95% confidence interval (CI), 81.5 to 100%], 99.2% analytical specificity (95% CI, 95.9 to 100%), and 100% diagnostic specificity (95% CI, 96.5 to 100%). This assay also allowed the detection of silent carriage of prions 1.3 and 2.6 years before the clinical onset in two blood donors who later developed vCJD. These data provide a key step toward the validation of this PMCA technology as a blood-based diagnostic test for vCJD and support its potential for detecting presymptomatic patients, a prerequisite for limiting the risk of vCJD transmission through blood transfusion.

INTRODUCTION

Variant Creutzfeldt-Jakob disease (vCJD) was identified in 1996 in the U.K. as a zoonotic infection caused by the dietary transmission of bovine spongiform encephalopathy (*1*, *2*). vCJD is a rare transmissible spongiform encephalopathy (TSE) with a long incubation period and with no validated test to identify affected individuals before clinical onset. Until recently, all clinical cases of vCJD evaluated for the prion protein gene (*PRNP*) were homozygous for methionine at codon 129 (59% of the Caucasian population) (*3*) with no cases in the alternative genotypes. The first confirmed vCJD clinical case in a patient heterozygous for methionine/valine has just been reported in the U.K. (*4*). Patients with vCJD display an accumulation of abnormally folded prion protein (PrP^{TSE}) in the brain and lymphoid tissues including the spleen, lymph nodes, appendix, and tonsils (*5*, *6*), raising concerns about the risk of blood-borne infection. The secondary transmission of prions through blood has been demonstrated in several animal models (*7*–*10*). In the U.K., four cases of infection by the vCJD agent have been reported in recipients of nonleukodepleted red blood cell concentrate from donors who were carriers for vCJD (*11*–*13*). A fifth probable case was reported in a patient with hemophilia treated with coagulation factor VIII manufactured from plasma derived from U.K. donors (*14*). Infectivity in blood is thought to be split about equally between leukocytes and plasma, with negligible amounts directly associated with red blood cells or platelets (*15*). Whereas clinical cases of vCJD are declining worldwide, the prevalence of asymptomatic carriers in the general population remains a concern. Investigations into the prevalence of asymptomatic carriers, using immunohistochemical detection of PrP^{TSE} in a large series of appendix specimens, indicated an unexpectedly high rate of infection (1/2000) in the general U.K. population (*16*, *17*), which can be extrapolated to a prevalence of 1/20,000 in France where the level of exposure is considered to be 10 times lower. Detecting circulating agents in asymptomatic subjects with peripheral prion infection is thus important for public health, in particular to avoid secondary transmission through blood transfusion. A prototype enzyme-linked immunosorbent assay (ELISA) test was recently reported to identify vCJD cases by probing whole blood (*18*–*20*). Several other strategies are being explored using blood (*21*–*23*) and urine from vCJD patients (*24*). However, most blood tests have only reached about 70% sensitivity in blinded studies, thus falling short of the acceptance criteria of 90% required by the European Union (EU) Commission Directive 2011/100/EU for licensing for human use (*25*). In addition, none of these tests has proven effective for detecting presymptomatic patients. We have previously described a blood diagnostic assay based on serial prion amplification by protein misfolding cyclic amplification (PMCA) (*26*). To circumvent the presence of blood-associated conversion inhibitors, we first captured the prion protein using magnetic nanobeads coated with plasminogen. Plasminogen was identified as a ligand of abnormal prion proteins using specific interactions with lysine residues by

[1]Etablissement Français du Sang, INSERM, Université de Montpellier, UMR 1058, TransDiag, F-34184 Montpellier, France. [2]Sorbonne Universités, UPMC Univ Paris 06, INSERM, CNRS, Institut du Cerveau et de la Moelle épinière (ICM)–Hôpital Pitié-Salpêtrière, F-75013 Paris, France. [3]Assistance publique–Hôpitaux de Paris, Cellule Nationale de Référence des Maladies de Creutzfeldt-Jakob, Groupe Hospitalier Pitié-Salpêtrière, F-75013 Paris, France. [4]Centre National de Référence des Agents Transmissibles Non Conventionnels, F-75013 Paris, France. [5]Institut National de la Recherche Agronomique, Université Paris-Saclay, Virologie Immunologie Moléculaire, F-78350 Jouy-en-Josas, France. [6]Laboratoire de Virologie, Hôpital Pellegrin, F-33076 Bordeaux, France. [7]Service de Biochimie et Biologie Moléculaire, Hôpital Lariboisière, F-75010 Paris, France. [8]National Creutzfeldt-Jakob Disease Research and Surveillance Unit, Centre for Clinical Brain Sciences, University of Edinburgh, Edinburgh EH4 2XU, U.K. [9]Etablissement Français du Sang, F-93218 La Plaine Saint Denis, France. [10]INSERM, Etablissement Français du Sang, Université de Franche-Comté, UMR 1098, F-25020 Besançon, France.
*Corresponding author. Email: joliette.coste@efs.sante.fr (J.C.); daisy.bougard@efs.sante.fr (D.B.)

Fischer *et al.* (*27*). Of particular interest is the capacity of plasminogen to bind to PrP^{TSE} from various species including humans as well as sporadic CJD (sCJD) prions (*28*). Plasminogen has also demonstrated the ability to stimulate prion conversion in vitro (*29*).

In our PMCA-based assay (*30*), the capture step enables the concentration of prion proteins present in 500 µl of plasma and their separation from blood-associated conversion inhibitors. Our assay has already achieved the level of performance required to detect PrP^{TSE} at the femtomolar range in plasma, buffy coat, or whole-blood samples using either citrate or EDTA as anticoagulant. Here, by testing rare specimens from U.K. and French CJD surveillance centers, we further assess the performance of our assay against the strict requirements outlined in the amended Decision 2002/364/EC of the European Directive 98/79/EC (*25*) regarding the common technical specifications for vCJD blood-screening assays. Our assay allowed us to diagnose in a blinded fashion the presence of PrP^{TSE} in samples from 18 of 18 symptomatic vCJD cases among a large sample collection, including samples from patients with sCJD, with 100% sensitivity. It also permitted PrP^{TSE} detection in plasma samples collected 14 and 31 months before clinical onset in two French blood donors who later developed vCJD.

RESULTS

Analytical sensitivity of the vCJD diagnostic assay

Our vCJD diagnostic assay consisted of a plasminogen-bead capture step followed by serial PMCA (Fig. 1). We validated our vCJD assay for analytical sensitivity (test detection threshold) using a blinded reference panel consisting of plasma (in citrate anticoagulant) spiked with World Health Organization (WHO) reference materials from the National Institute for Biological Standards and Control (NIBSC) (NHBY0/0003 for brain and NHSY0/0009 for spleen from vCJD patients) (*30*).To refine these sensitivity data, we prepared an additional panel of human plasma samples spiked with serial dilutions of the same vCJD WHO reference brain tissue ranging from 10^{-5} to 10^{-12}. After three PMCA rounds of 80 cycles each, PrP^{TSE} could be detected at a dilution of 10^{-9} (Fig. 2). Additional rounds of PMCA did not increase this level of analytical sensitivity. The number of misfolded PrP^{TSE} molecules in the 10^{-10} dilution of the brain (and lower dilutions) and recovered by the plasminogen beads as the PMCA seed input was likely insufficient to initiate the amplification process. However, this 10^{-9} dilution of analytical sensitivity is 4 to 5 log higher than the acceptance criteria of 10^{-4} dilution required by the Common Technical Specifications defined in the European Commission Directive regarding the performances of vCJD screening assays (*25*).

Diagnostic sensitivity and analytical specificity of the vCJD assay

To evaluate the diagnostic sensitivity and the analytical specificity of our assay, we analyzed in a blinded fashion a total of 152 plasma samples from French and British patients, including 18 patients with vCJD, 67 with sCJD, and 67 with non-CJD neurological diseases. In addition, plasma samples from 104 blood donors were tested as healthy controls to evaluate diagnostic specificity (Table 1).

Among the 152 plasma samples from patients, our assay blindly identified all 18 cases of clinical vCJD, thus achieving a diagnostic sensitivity of 100% [95% confidence interval (CI), 81.5 to 100]. As shown in Table 2, of the 18 vCJD samples, 1 was positive after two rounds of PMCA, 8 were positive after three rounds of PMCA, and 9 were positive after four rounds. Whereas the detection limit was reached after three rounds of PMCA for brain material spiked into the plasma (exogenous prions), one additional round of PMCA increased the sensitivity of the assay when plasma samples from vCJD patients (endogenous prions) were tested. Blood samples were obtained for all patients shortly before death, except for five that were collected at earlier clinical phases of the disease. The number of rounds required to obtain a positive signal showed no correlation with the time at which the tested sample was collected before the patient's death.

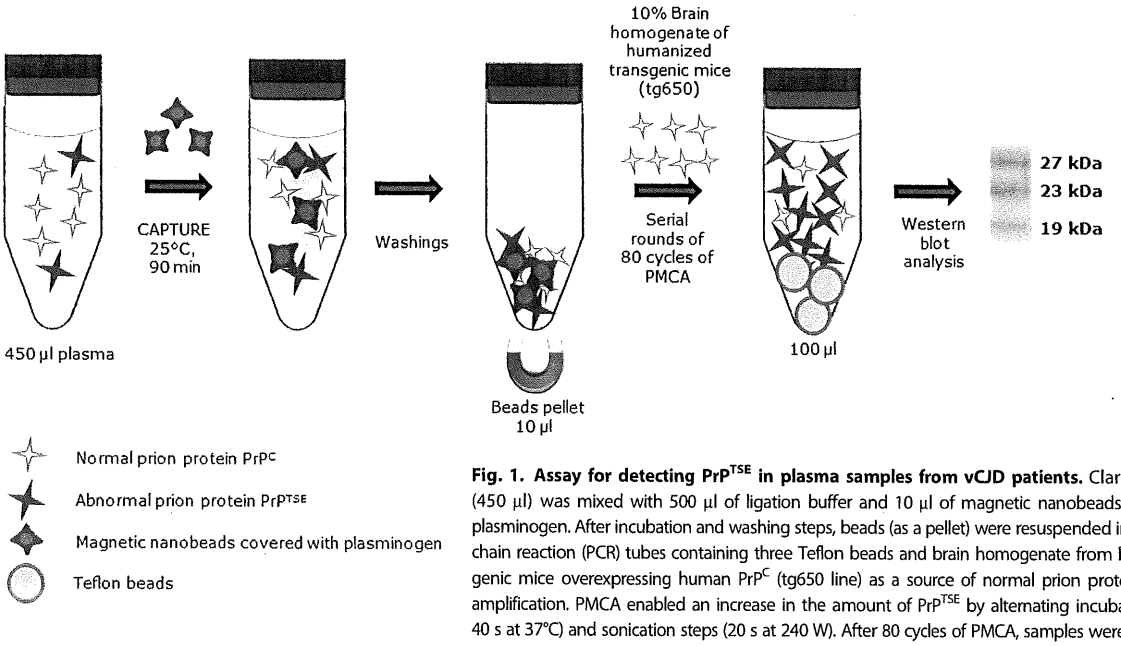


Fig. 1. Assay for detecting PrP^{TSE} in plasma samples from vCJD patients. Clarified plasma (450 µl) was mixed with 500 µl of ligation buffer and 10 µl of magnetic nanobeads coated with plasminogen. After incubation and washing steps, beads (as a pellet) were resuspended in polymerase chain reaction (PCR) tubes containing three Teflon beads and brain homogenate from healthy transgenic mice overexpressing human PrP^{C} (tg650 line) as a source of normal prion protein for PrP^{TSE} amplification. PMCA enabled an increase in the amount of PrP^{TSE} by alternating incubation (29 min 40 s at 37°C) and sonication steps (20 s at 240 W). After 80 cycles of PMCA, samples were analyzed by Western blot and/or subjected to a subsequent round of PMCA by refreshing the substrate.

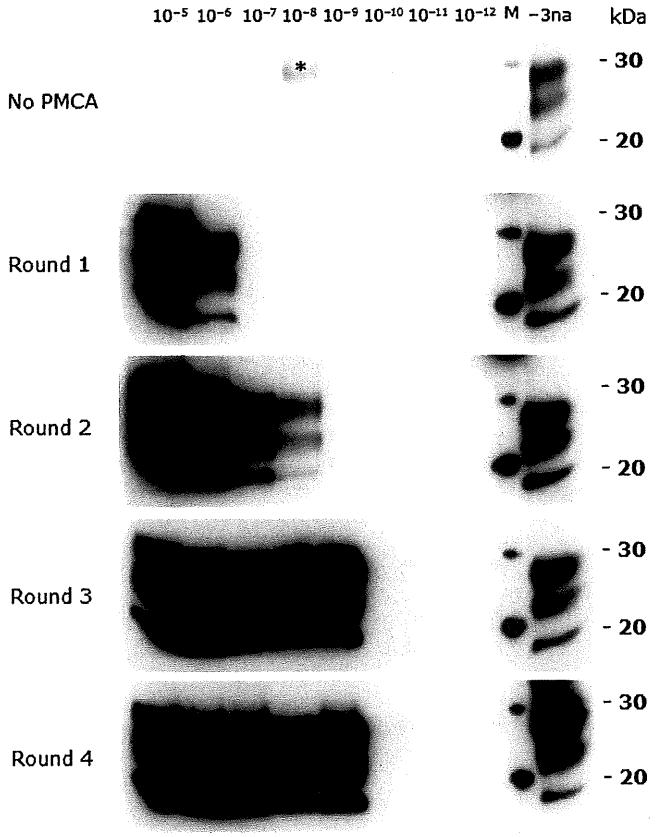


Fig. 2. Analytical sensitivity of the vCJD detection assay. The analytical sensitivity of the assay was assessed by analyzing plasma samples supplemented with log dilutions of vCJD reference brain tissue ranging from 10^{-5} to 10^{-12}. The PrP^{TSE} signal was assessed by means of Western blot analysis using the 3F4 antibody after proteinase K digestion. For each sample, 20 µl of the product was loaded onto the gel. As a Western blot control, a 10^{-3} dilution of the vCJD reference brain sample without any amplification (no PMCA) was loaded on each gel (−3na). The results obtained after each serial round of PMCA are shown. The asterisk indicates a faint signal from incomplete PrP^{C} digestion. M, molecular weight markers.

As shown in Table 1, our assay also showed high analytical specificity, with only 1 of 134 potentially cross-reacting blood specimens from patients with sCJD, Alzheimer's disease, Parkinson's disease, and other neurological diseases giving a positive result (99.2% specificity; 95% CI, 95.9 to 100). The positive specimen was from a 65-year-old patient in the U.K. with sCJD (homozygous for methionine at codon 129 with a 2a molecular typing; type MM2a). Two other plasma samples from type MM2a sCJD patients were tested in this study and gave negative results for PrP^{TSE}. Results obtained on the whole U.K. panel (20 plasma samples) are shown in Fig. 3. Representative results obtained on French plasma samples are shown in Fig. 4A. No PrP^{TSE} signal was detected in the 104 plasma samples from blood donors (Table 1) even after five rounds of PMCA, thus indicating a diagnostic specificity of 100% (95% CI, 96.5 to 100).

We then compared by Western blotting the PrP^{TSE} molecular signature obtained for the clinical vCJD amplified samples with that of the reference brain samples from patients with sCJD or vCJD (Figs. 3 and 4B). Samples from all the vCJD patients displayed the characteristic type 2 mobility (19 kDa) and clear predominance of the diglycosylated isoform (27 kDa), whether amplified from plasma (Fig. 4B; vCJD plasma) or from crude brain tissue (Fig. 4B; vCJD brain homogenate). A similar type 2 mobility was also seen on the amplified products obtained from the U.K. patient samples (Fig. 3), including the 10 vCJD samples and strikingly with the sCJD patient classified as MM2a [UK-14; Fig. 3].

Preclinical detection of abnormal prions in plasma

In France, since 1999, as an additional control for blood safety, two 500-µl nonleukodepleted aliquots of the plasma of all blood donations are archived in liquid nitrogen for 5 years by the Etablissement Français du Sang (French blood service). Three of the 27 patients who developed clinical signs of vCJD had been regular blood donors (cases 8, 9, and 13). Eleven sequential archived plasma vials from two of these three donors were traced and specifically archived for long-term conservation. These vials correspond to the seven last blood donations from donor A (case 8) and the four last donations from donor B (case 13). No archived samples could be traced for case 9. Donors A and B donated once or twice a year before they developed clinical signs of vCJD 7 and 2 months, respectively, after their last blood donation. Thus, we analyzed 11 nonleukodepleted plasma samples from donors A and B starting from 55 to 30 months, respectively, before clinical symptom onset. For donor A (Fig. 5A), although the first four plasma samples (A1 to A4) were negative after six successive rounds of PMCA, we detected PrP^{TSE} 31 months before clinical onset (A5) and in subsequent blood donations (A6 and A7) after three or four PMCA rounds. In donor B, after six PMCA rounds, PrP^{TSE} detection was negative 30 months before clinical onset (B1) and became positive 14 months later (B2), which was 16 months before the appearance of clinical signs of the disease (Fig. 5B). Subsequent blood donations (B3 and B4) were also positive for vCJD prions after three or four PMCA rounds. These results demonstrate the presence of PrP^{TSE} in the plasma of individuals during the presymptomatic phase of vCJD.

DISCUSSION

Here, we report a detection method in plasma allowing the diagnosis of clinical vCJD with 100% sensitivity, 99.2% analytical specificity, and 100% diagnostic specificity. Despite the rarity of the samples, we have tested 18 blood samples from vCJD patients from France and the U.K. Eighteen of 18 clinical vCJD cases were identified in a blinded fashion. Such results satisfy the requirements for both sensitivity and specificity mandated by the European Commission Directive (*25*). In a related study in this issue, Concha-Marambio *et al.* provided further support for the validity of the PMCA technology for detecting vCJD in human blood samples with 100% sensitivity and specificity (*31*).

The finding that 1 of 67 sCJD patients tested positive for PrP^{TSE} in plasma may suggest that our assay could sporadically amplify type MM2a PrP^{TSE} under conditions optimized for vCJD detection. The presence of infectivity in the blood of patients with sCJD has recently been reported (*32*) but requires further investigation. Jackson *et al.* (*20*), when determining the diagnostic performance of their prototype vCJD ELISA blood test, also found two patients with sCJD who tested positive, indicating some reactivity of their assay for sCJD in whole blood. Lastly, and as suggested by the results obtained in prevalence studies on appendix tissue (*16*), we cannot exclude the possibility that the MM2a U.K. sCJD patient testing positive in our assay was also a carrier of vCJD infection in lymphoid tissue. No peripheral tissues are available from this case to investigate this hypothesis.

Table 1. Analytical performance of the plasminogen-based capture and amplification assay.

	Diagnosis	Patients with positive detection of Pr^{TSE} in plasma samples. No./total no.	Analytical performance, % (95% CI)
Clinical CJD	vCJD	18/18	Diagnostic sensitivity, 100% (81.5 to 100)
	Probable	4/4	
	Definite	14/14	
	sCJD	1/67	Analytical specificity, 99.2% (95.9 to 100)
	Probable	0/27	
	Definite	1*/40	
Non-CJD	Other neurodegenerative diseases	0/15	
	Alzheimer's disease	0/9	
	Lewy body dementia	0/3	
	Parkinson's disease	0/2	
	Frontotemporal dementia	0/1	
	Other nonneurodegenerative diseases	0/52	
	Metabolic and toxic encephalopathies	0/14	
	Paraneoplastic encephalitis and cancer	0/12	
	Neurovascular disease	0/7	
	Infectious diseases	0/6	
	Autoimmune encephalopathies	0/3	
	Other disorders**	0/10	
Blood donors	Healthy controls	0/104	Diagnostic specificity, 100% (96.5 to 100)
	Presymptomatic vCJD***	2/2	

*U.K. patient aged 65 years, sCJD type MM2a. **Epilepsy (n = 2), brain anoxia (n = 2), psychiatric diseases (n = 2), celiac disease (n = 1), cerebellar ataxia (n = 1), confusion (n = 1), and polyradiculoneuropathy (n = 1). ***Both patients are distinct from the eight French patients with clinical vCJD sampled during the clinical course of the disease.

Using recombinant PrP as a substrate instead of brain homogenate and intensive agitation instead of sonication, an alternative method called real-time quaking-induced conversion (RT-QuIC) has emerged as a powerful tool for prion detection (*33*, *34*). Independent studies have shown that the detection in the cerebrospinal fluid or in the olfactory epithelium of PrP^{TSE} for the diagnosis of sCJD by RT-QuIC may be 99 to 100% specific (*35*–*37*). However, as yet, RT-QuIC appears to be less efficient in detecting PrP^{TSE} from vCJD patients using various full-length recombinant PrP (*38*), although the use of hamster-sheep chimeric PrP may improve its sensitivity (*22*). From a clinical point of view, to date, RT-QuIC–derived methods have not been used to diagnose vCJD. Two detection assays have been described for the detection of vCJD prions in blood with sensitivities close to 70%. The first one combines a solid-state binding matrix to capture and concentrate PrP^{TSE} from whole-blood samples with direct immunodetection (*18*). Having demonstrated 98.1% analytical specificity (95% CI, 93.3 to 99.8%) with two sCJD positives detected and 100% diagnostic specificity (95% CI, 99.9 to 100%) in 5000 U.S. blood donor samples (*20*), this assay appears promising as a screening assay. The second assay is a PMCA-based assay that has allowed the detection of PrP^{TSE} in white blood cells from three of four patients with clinical vCJD (*21*). The diagnosis of vCJD may also be achieved using urine samples according to a third PMCA-based assay (*24*), which has also shown promising results: 92.9% sensitivity on 14 symptomatic vCJD patient samples (95% CI, 66.1 to 99.8%) and 100% diagnostic specificity on 52 healthy control samples (95% CI, 93.2 to 100%). There is no evidence that these assays can detect PrP^{TSE} in carriers incubating the disease, who have not yet shown clinical symptoms.

Here, we report the identification of asymptomatic patients with detectable PrP^{TSE} in the blood before clinical onset. The observed kinetics of the appearance of detectable PrP^{TSE} in plasma indicates that a presymptomatic diagnosis is possible up to 31 months before the first clinical symptoms appear. This is consistent with the intervals of 17 to 40 months between the blood donations most likely responsible for secondary vCJD transmission in the U.K. and the onset of vCJD symptoms observed in the corresponding donors (*11*, *13*, *39*). The number of PMCA rounds required to obtain a positive signal varied between three and four during the incubation period in each

asymptomatic case and between two and four after clinical onset. The kinetics of circulating PrP^{TSE} during the incubation period and during the clinical phase of the natural disease is unknown in humans. It remains unclear whether circulating PrP^{TSE} continuously increases during the incubation period and whether it reaches a steady state during the clinical phase. It is worth noting that the presence of PrP^{TSE} can be scarce in the lymphoid tissues of some symptomatic vCJD patients, leading to false negatives on tonsil biopsy (*3*, *40*). Our results suggest that circulating PrP^{TSE} during incubation and disease usually fluctuates within the limits of detection corresponding to three or four rounds of our PMCA assay, which is equivalent to the PrP^{TSE} contained in 2 to 20 pg of vCJD brain tissue. A quantitative PMCA assay as described by Chen *et al.* (*41*) may be a useful approach to precisely assess PrP^{TSE} concentrations in the blood of vCJD patients.

Table 2. Characteristics of the 18 vCJD clinical cases included in this study.

Case no.	Duration of the clinical phase (months)	Time at which the tested sample was harvested (months before death)	No. of rounds of serial PMCA to detect PrP^{TSE} in the plasma samples
FR-20	14.0	2.6	3
FR-21	8.7	2.8	3
FR-22	15.8	9.8	3
FR-23	9.8	1.9	4
FR-24	13.5	5.5	3
FR-25	13.4	5.5	3
FR-26	26.7	10.1	3
FR-27	27.1	21.2	4
UK-1	14	4.3	4
UK-2	15	10.5	3
UK-5	14	0.9	3
UK-6	10	3.4	4
UK-8	18	9.7	4
UK-11	18	6.0	4
UK-15	13	2.8	2
UK-16	24	0.4	4
UK-17	10	6.7	4
UK-19	15	2.0	4

Our findings demonstrate that measurable amounts of PrP^{TSE} with seeding activity can be found in the blood of humans at a presymptomatic stage. This has profound implications for public health and risk management, particularly in transfusion medicine. Our results provide a first estimation of the level of circulating abnormal prions in the plasma of patients in the final years of the incubation period for vCJD, which is not dissimilar from that observed in symptomatic patients. In France, no case of transfusion-transmitted vCJD has been reported so far in patients treated with blood products from the three vCJD patients who donated before symptom onset. This observation suggests that the precautionary measures introduced in France to safeguard blood transfusion, in particular leukodepletion, may have reduced the risk of transmission. With a limit of detection in the femtomolar range, our assay should allow a more precise evaluation of the residual risk of vCJD prion distribution in the different blood components. It may also be well suited for use as a complementary confirmatory assay, ensuring that any positive results from high-throughput blood-screening assays are true positives.

From a clinical point of view, a diagnostic method with 100% sensitivity is of special interest for diagnosis in young patients showing clinical signs that do not fulfill the criteria of probable vCJD or in older patients with atypical forms of rapidly progressive dementia (*42*).

There are some limitations to our study. Only a limited number of blood samples from vCJD patients with clinical disease were tested, but the number of samples in this study nonetheless fulfilled the requirements of the European Commission Directive (*25*) because vCJD is a very rare disease. In addition, the number of 18 symptomatic vCJD patients is within the range of recent international studies in the field of vCJD diagnosis (*18*, *24*). Our study demonstrated the presence of PrP^{TSE} in the plasma in only two presymptomatic vCJD cases, but samples of such presymptomatic blood donors are rare, and their

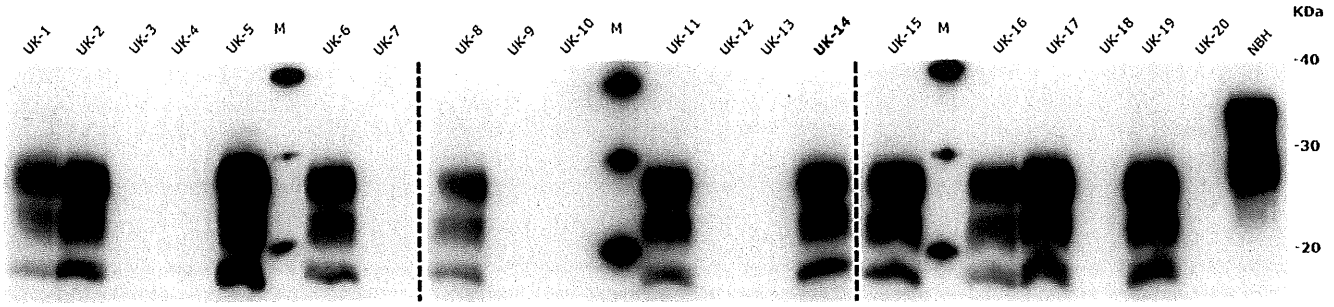


Fig. 3. Detection of PrP^{TSE} in plasma samples from U.K. patients with CJD. Blinded plasma samples obtained from 20 U.K. CJD patients were analyzed. The PrP^{TSE} signal was assessed by means of Western blot analysis using 3F4 antibody after proteinase K digestion. For each positive signal, the equivalent of 7 to 10 μl of the product obtained after four rounds of PMCA was loaded onto the gel, whereas for negative signals, 20 μl of the product obtained after five rounds of PMCA was loaded onto the gel. UK-1, UK-2, UK-5, UK-6, UK-8, UK-11, UK-15, UK-16, UK-17, and UK-19 refer to vCJD patients. UK-3, UK-4, UK-7, UK-9, UK-10, UK-12, UL-13, UK-14, UK-18, and UK-20 refer to sCJD patients. NBH refers to a negative control brain homogenate from a non-CJD individual without any proteinase K digestion. M, molecular weight marker. The dashed lines indicate separate Western blots.

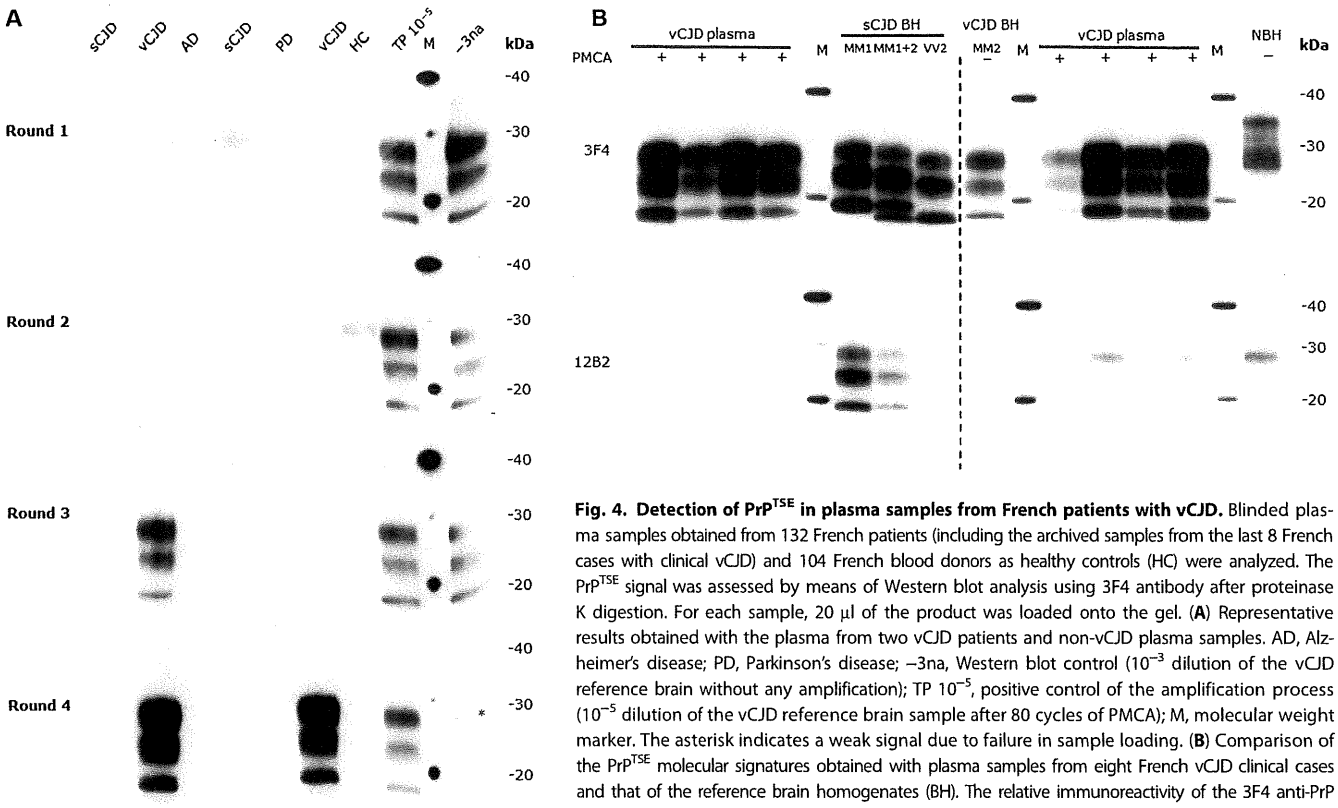


Fig. 4. Detection of PrP^{TSE} in plasma samples from French patients with vCJD. Blinded plasma samples obtained from 132 French patients (including the archived samples from the last 8 French cases with clinical vCJD) and 104 French blood donors as healthy controls (HC) were analyzed. The PrP^{TSE} signal was assessed by means of Western blot analysis using 3F4 antibody after proteinase K digestion. For each sample, 20 μl of the product was loaded onto the gel. (**A**) Representative results obtained with the plasma from two vCJD patients and non-vCJD plasma samples. AD, Alzheimer's disease; PD, Parkinson's disease; –3na, Western blot control (10^{-3} dilution of the vCJD reference brain without any amplification); TP 10^{-5}, positive control of the amplification process (10^{-5} dilution of the vCJD reference brain sample after 80 cycles of PMCA); M, molecular weight marker. The asterisk indicates a weak signal due to failure in sample loading. (**B**) Comparison of the PrP^{TSE} molecular signatures obtained with plasma samples from eight French vCJD clinical cases and that of the reference brain homogenates (BH). The relative immunoreactivity of the 3F4 anti-PrP monoclonal antibody (epitope amino acids 109 to 112) and the 12B2 anti-PrP monoclonal antibody (epitope amino acids 89 to 93) is shown. 12B2 is specific for type 1 PrP^{TSE} because its epitope is cleaved during proteinase K digestion in type 2 PrP^{TSE}. NBH refers to a negative control brain homogenate from a non-CJD plasma sample without any proteinase K digestion. The dashed line indicates separate Western blots.

inclusion provided a unique opportunity to assess the kinetics of PrP^{TSE} presence in blood during the incubation period. Until recently, all patients with clinical vCJD worldwide, including those enrolled in the present study (table S1), have been methionine-homozygous at codon 129 of the *PRNP* gene. The potential for infection to occur in lymphoid tissues and in the central nervous system in the other genotypes at codon 129 is supported by the observation of PrP^{TSE} accumulation in the lymphoid tissues of an asymptomatic heterozygous recipient of a potentially contaminated blood transfusion (*12*) and the recent report of the first vCJD case in a heterozygous patient (*4*). In addition, 50% of the asymptomatic carriers identified in the prevalence study on appendix tissues (*16*) were not homozygous for methionine at codon 129. Although the present study is an important first step for the systematic detection of infected individuals, with a significant potential impact on transfusion safety, whether the assay could detect PrP^{TSE} in the blood of vCJD patients with other genotypes has yet to be confirmed.

In conclusion, our results provide valuable data on circulating abnormal prion proteins during the incubation phase in humans and validate a new protein misfolding amplification–based assay that will reduce the potential risk of vCJD secondary transmission. Moreover, beyond these public health issues, our study provides evidence that a brain proteinopathy responsible for neurodegenerative disease can be diagnosed in asymptomatic patients through the detection of circulating abnormal protein aggregates. This finding is of interest for future translational research in neurological diseases caused by protein misfolding and aggregation, such as Alzheimer's disease and Parkinson's disease, for which there is a major need for a noninvasive early diagnostic test.

MATERIALS AND METHODS

Study design

The aim of the study was to investigate the suitability of our blood diagnostic assay comprising plasminogen-bead capture coupled with PMCA to identify vCJD-infected individuals. Compared with the handling of an emerging viral infection, development of a screening assay for infectious prions that in addition can be applied to blood samples from asymptomatic patients is challenging. In particular, only a small number of blood samples are available from clinical cases in France; no seroconversion panels are available nor a gold standard assay against which candidate-screening assays can be compared. From a regulatory point of view, vCJD assays for blood screening, diagnosis, and confirmation have been added to List A of Annexe II to Directive 98/79/EC by the Commission Directive 2011/100/EU of the EU In Vitro Diagnostics Directive, meaning that candidate assays must meet strict requirements with regard to sensitivity and specificity (*25*). Our study aimed to meet these acceptance criteria by testing plasma samples distributed in blinded panels from the U.K. and France. These panels included 18 plasma samples from vCJD patients, 134 potentially cross-reacting plasma samples from patients with neurological disorders, and 104 plasma samples from French blood donors. The detectability

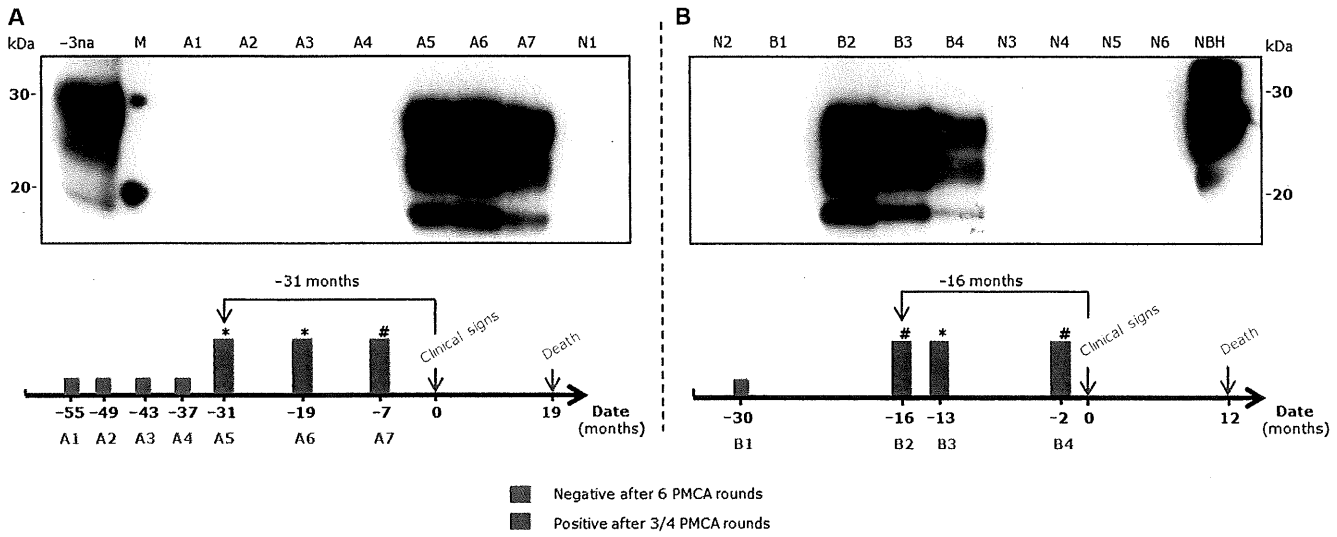


Fig. 5. Detection of PrP^{TSE} in plasma from two blood donors in the preclinical phase of vCJD. A set of 20 archived plasma vials stored at the Etablissement Français du Sang was tested. Seven plasma samples (labeled A1 to A7) had been collected from the eighth vCJD French case (donor A) during each of his last blood donations before he developed clinical signs of disease. Similarly, four plasma samples (labeled B1 to B4) had been collected from the 13th vCJD French case (donor B). Time of plasma sample collection (months) for each case is shown as the dates at which the first clinical signs appeared. Nine plasma control samples were obtained from blood donors who never developed CJD. Results obtained for all the plasma samples from donor A (**A**) and donor B (**B**) and six control plasma samples (N1 to N6) are shown. The PrP^{TSE} signal was assessed by means of Western blot analysis with 3F4 antibody after proteinase K digestion. For each sample, 20 μl of the product obtained after six rounds of PMCA was loaded onto the gel, except for samples labeled A5, A6, A7, B2, B3, and B4 for which the equivalent of 4 μl of the product obtained after four rounds of PMCA was loaded. −3na, Western blot control (10^{-3} dilution of the vCJD reference brain tissue without any amplification); NBH, a negative control brain homogenate from a non-CJD plasma sample without any proteinase K digestion; M, molecular weight markers. "*" indicates positive after three rounds and "#" indicates positive after four rounds.

of PrP^{TSE} in the blood of presymptomatic carriers was also assessed on 11 archived plasma vials from two blood donors who subsequently developed vCJD.

Normal plasma samples and reference brain tissues

To assess the diagnostic specificity, anonymized blood samples were collected from consenting French blood donors in compliance with French regulations (code de la santé publique article L.1243-3). Whole-blood samples (n = 104) were collected in EDTA-containing tubes. Plasma was isolated after centrifugation at 1500g for 15 min at 21°C and stored at −80°C until use.

For the study of analytical sensitivity, WHO reference brain samples (10%, w/v) were provided by the NIBSC (U.K.) under the reference number NHBY0/0003 for vCJD and number NHBX0/0001 for sCJD [methionine-homozygous at codon 129 with PrP^{TSE} type 1 + 2 (MM1 + 2)]. Two additional sCJD brain samples were provided by CHU-Lyon (France), including one methionine-homozygous case with PrP^{TSE} type 1 (MM1) and one valine-homozygous case with PrP^{TSE} type 2 (VV2). NIBSC also provided a negative control WHO reference brain homogenate (number NHBZ0/0005).

Blind panels from the French CJD surveillance network

Since 1993, all CJD suspects across France have been notified to the national CJD surveillance network (*43*). Most suspects are notified by the laboratories that perform cerebrospinal fluid examination for detection of the 14-3-3 protein. Other sources of notification are neurological clinics, neuropathology laboratories, and the French national institute for public health surveillance [Institut de Veille Sanitaire (InVS)], which collects data on all notifiable diseases. For each suspected case, the CJD surveillance network collects all available medical data to classify each case as sCJD, genetic CJD, iatrogenic CJD, vCJD (definite, probable, or possible), or non-CJD, using internationally recognized criteria (*44*, *45*). Only definite and probable CJD cases are reported to the EuroCJD network, and all vCJD cases are reported to the European Centre for Disease Prevention and Control.

Blood sampling from cases with a suspected diagnosis of CJD notified to the surveillance network was initiated in June 2006. This collection is dedicated to the assessment of novel diagnostic procedures for CJD. An informed and signed consent from the patients or their relatives was obtained for each sample. The procedure was approved by the ethics committee of the Pitié-Salpêtriere Hospital (CCPPRB/130-05). Blood was sampled in tubes containing sodium citrate, heparin, or EDTA. After centrifugation at 2000g for 15 min at 4°C, plasma, buffy coat, and red blood cells were sampled in aliquots of 150 μl and stored at −80°C. Here, only EDTA plasma samples were used. Samples from 132 patients including 8 cases with vCJD (the last 8 cases on a total of 27 French cases who have died of vCJD), 57 cases with sCJD, and 67 cases with other neuropsychiatric diagnosis (see Table 1) were split up into six consecutive groups and provided blindly to undergo our testing procedure.

Blind panels from the U.K. National CJD Research and Surveillance Unit

The National CJD Research and Surveillance Unit was established in 1990 and receives referrals of all suspect cases of CJD in the U.K. Whenever possible, staff from the unit visit the referred cases for assessment and collection of detailed medical data before classifying the cases using internationally recognized criteria (*44*, *45*). Only definite and probable CJD cases are reported to the EuroCJD network, and

all vCJD cases are reported to the European Centre for Disease Prevention and Control.

Blood samples have been obtained in all cases since 1990. Informed and signed consent was provided by the patient or relatives. The study was approved by the National Health Service Lothian Ethics Committee. The blood samples were collected in EDTA or citrate. After centrifugation at 400*g* for 10 min, plasma, buffy coat, and red blood cells were stored at −80°C. Here, samples stored in citrate or EDTA were used. In eight vCJD samples, the type of anticoagulant, either EDTA or citrate, was not recorded. Samples included 10 from vCJD cases (of 178 cases of vCJD in the U.K.) and 10 from sCJD cases. The 20 samples were provided blinded to undergo the testing procedure.

Archived plasma panels from the Etablissement Français du Sang (French blood service)

Since 1999, two aliquots of nonleukodepleted plasma from all blood donations are archived for 5 years by the Etablissement Français du Sang in liquid nitrogen to allow additional controls with regard to blood safety. These aliquots, each of 500 µl, are prepared from whole blood collected in EDTA-containing tubes. Eleven sequential archived plasma vials from two donors who later developed vCJD were traced and specifically archived for long-term conservation (over 5 years). These vials correspond to the last seven blood donations for the 8th vCJD French case (donor A) and the last four donations for the 13th vCJD French case (donor B). Nine archived plasma samples from blood donors who never developed CJD were added to this study.

Testing procedure

The testing procedure is a three-step assay (*30*) that captures PrP^{TSE} from infected blood using plasminogen-coated magnetic nanobeads before serial amplification of PrP^{TSE} via PMCA and finally specific detection by Western blot (Fig. 1). Plasma samples were thawed in a water bath for 10 min at 37°C and clarified at 1500*g* for 5 min at 21°C. In the preanalytical capture step, 10 µl of 1% (w/v) beads (Ademtech) at 10 µg of plasminogen per milligram of beads was added to a mix (1:1) of plasma samples (400 to 500 µl) and ligation buffer [0.1 M phosphate, 0.5% sodium chloride, and 0.1% sarkosyl (pH 7.4)] before being incubated for 90 min at 25°C. After washing steps with phosphate-buffered saline, beads were directly resuspended in the PMCA substrate.

For the PMCA amplification, the source of PrP used as substrate was obtained from brain homogenates of healthy transgenic mice overexpressing sixfold more human PrP with a methionine at codon 129 (tg650 line) (*46*). Brains were prepared at 10% (w/v) homogenate in converting buffer (phosphate-buffered saline containing 150 mM sodium chloride and 1% Triton) and clarified at 2000*g* for 20 s before freezing at −80°C in single-experiment aliquots of PMCA substrate.

The captured prion protein was mixed with 90 µl of PMCA substrate in PCR tubes containing three Teflon beads. Amplification (80 cycles) was performed by three different technicians using two different types of sonicators (S4000, Misonix, and Q700; Qsonica). Each cycle is composed of an incubation step (29 min 40 s at 37°C) and a sonication step (20 s at 240 W). Successive rounds of 80 cycles were performed by diluting the amplified material 1:10 in fresh PMCA substrate. To avoid any cross-contamination, experiments were carried out under strict quality-controlled PCR conditions. In addition, positive samples were not systematically subjected to an additional PMCA round.

After amplification, protease-resistant prion protein was detected by Western blot, as previously described (*47*). Samples were incubated with proteinase K (200 µg/ml) for 60 min at 45°C before denaturation at 100°C in denaturing buffer. Samples were run on 12% polyacrylamide gel electrophoresis before being electrotransferred onto a polyvinylidene difluoride membrane and revealed using 3F4 or 12B2 monoclonal antibodies against prion protein.

Statistical analysis

Calculations of 95% Clopper-Pearson CIs for sensitivities and specificities were performed according to the exact binomial test (*48*) using the free software R (*49*).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/8/370/370ra182/DC1

Methods

Table S1. Codon 129 genotype of the patients included in this study (including presymptomatic cases).

Reference (*50*)

REFERENCES AND NOTES

1. M. E. Bruce, R. G. Will, J. W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser, C. J. Bostock, Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**, 498–501 (1997).
2. R. G. Will, J. W. Ironside, M. Zeidler, S. N. Cousens, K. Estibeiro, A. Alperovitch, S. Poser, M. Pocchiari, A. Hofman, P. G. Smith, A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347**, 921–925 (1996).
3. J.-P. Brandel, C. A. Heath, M. W. Head, E. Levavasseur, R. Knight, J.-L. Laplanche, J. P. M. Langeveld, J. W. Ironside, J.-J. Hauw, J. Mackenzie, A. Alpérovitch, R. G. Will, S. Haïk, Variant Creutzfeldt–Jakob disease in France and the United Kingdom: Evidence for the same agent strain. *Ann. Neurol.* **65**, 249–256 (2009).
4. National Creutzfeldt-Jakob Disease Research and Surveillance Unit, *Variant Creutzfeldt-Jakob Disease Cases Worldwide* (2016); www.cjd.ed.ac.uk/documents/worldfigs.pdf.
5. A. F. Hill, R. J. Butterworth, S. Joiner, G. Jackson, M. N. Rossor, D. J. Thomas, A. Frosh, N. Tolley, J. E. Bell, M. Spencer, A. King, S. Al-Sarraj, J. W. Ironside, P. L. Lantos, J. Collinge, Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet* **353**, 183–189 (1999).
6. J. D. F. Wadsworth, S. Joiner, A. F. Hill, T. A. Campbell, M. Desbruslais, P. J. Luthert, J. Collinge, Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet* **358**, 171–180 (2001).
7. O. Andréoletti, C. Litaise, H. Simmons, F. Corbière, S. Lugan, P. Costes, F. Schelcher, D. Vilette, J. Grassi, C. Lacroux, Highly efficient prion transmission by blood transfusion. *PLOS Pathog.* **8**, e1002782 (2012).
8. N. Bons, S. Lehmann, N. Mestre-Francès, D. Dormont, P. Brown, Brain and buffy coat transmission of bovine spongiform encephalopathy to the primate *Microcebus murinus*. *Transfusion* **42**, 513–516 (2002).
9. F. Houston, J. D. Foster, A. Chong, N. Hunter, C. J. Bostock, Transmission of BSE by blood transfusion in sheep. *Lancet* **356**, 999–1000 (2000).
10. C. Lacroux, D. Bougard, C. Litaise, H. Simmons, F. Corbiere, D. Dernis, R. Tardivel, N. Morel, S. Simon, S. Lugan, P. Costes, J. L. Weisbecker, F. Schelcher, J. Grassi, J. Coste, O. Andréoletti, Impact of leucocyte depletion and prion reduction filters on TSE blood borne transmission. *PLOS ONE* **7**, e42019 (2012).
11. C. A. Llewelyn, P. E. Hewitt, R. S. G. Knight, K. Amar, S. Cousens, J. Mackenzie, R. G. Will, Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* **363**, 417–421 (2004).
12. A. H. Peden, M. W. Head, D. L. Ritchie, J. E. Bell, J. W. Ironside, Preclinical vCJD after blood transfusion in a *PRNP* codon 129 heterozygous patient. *Lancet* **364**, 527–529 (2004).
13. S. J. Wroe, S. Pal, D. Siddique, H. Hyare, R. Macfarlane, S. Joiner, J. M. Linehan, S. Brandner, J. D. F. Wadsworth, P. Hewitt, J. Collinge, Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: A case report. *Lancet* **368**, 2061–2067 (2006).
14. A. Peden, L. McCardle, M. W. Head, S. Love, H. J. T. Ward, S. N. Cousens, D. M. Keeling, C. M. Millar, F. G. H. Hill, J. W. Ironside, Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia. *Haemophilia* **16**, 296–304 (2010).
15. K. Holada, J. G. Vostal, P. W. Theisen, C. MacAuley, L. Gregori, R. G. Rohwer, Scrapie infectivity in hamster blood is not associated with platelets. *J. Virol.* **76**, 4649–4650 (2002).

16. O. N. Gill, Y. Spencer, A. Richard-Loendt, C. Kelly, R. Dabaghian, L. Boyes, J. Linehan, M. Simmons, P. Webb, P. Bellerby, N. Andrews, D. A. Hilton, J. W. Ironside, J. Beck, M. Poulter, S. Mead, S. Brandner, Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: Large scale survey. *BMJ* **347**, f5675 (2013).
17. D. A. Hilton, A. C. Ghani, L. Conyers, P. Edwards, L. McCardle, D. Ritchie, M. Penney, D. Hegazy, J. W. Ironside, Prevalence of lymphoreticular prion protein accumulation in UK tissue samples. *J. Pathol.* **203**, 733–739 (2004).
18. J. A. Edgeworth, M. Farmer, A. Sicilia, P. Tavares, J. Beck, T. Campbell, J. Lowe, S. Mead, P. Rudge, J. Collinge, G. S. Jackson, Detection of prion infection in variant Creutzfeldt-Jakob disease: A blood-based assay. *Lancet* **377**, 487–493 (2011).
19. G. S. Jackson, J. Burk-Rafel, J. A. Edgeworth, A. Sicilia, S. Abdilahi, J. Korteweg, J. Mackey, C. Thomas, G. Wang, S. Mead, J. Collinge, A highly specific blood test for vCJD. *Blood* **123**, 452–453 (2014).
20. G. S. Jackson, J. Burk-Rafel, J. A. Edgeworth, A. Sicilia, S. Abdilahi, J. Korteweg, J. Mackey, C. Thomas, G. Wang, J. M. Schott, C. Mummery, P. F. Chinnery, S. Mead, J. Collinge, Population screening for variant Creutzfeldt-Jakob disease using a novel blood test: Diagnostic accuracy and feasibility study. *JAMA Neurol.* **71**, 421–428 (2014).
21. C. Lacroux, E. Comoy, M. Moudjou, A. Perret-Liaudet, S. Lugan, C. Litaise, H. Simmons, C. Jas-Duval, I. Lantier, V. Béringue, M. Groschup, G. Fichet, P. Costes, N. Streichenberger, F. Lantier, J. P. Deslys, D. Vilette, O. Andréoletti, Preclinical detection of variant CJD and BSE prions in blood. *PLOS Pathog.* **10**, e1004202 (2014).
22. C. D. Orrú, J. M. Wilham, L. D. Raymond, F. Kuhn, B. Schroeder, A. J. Raeber, B. Caughey, Prion disease blood test using immunoprecipitation and improved quaking-induced conversion. *MBio* **2**, e00078-11 (2011).
23. P. Saá, J. Castilla, C. Soto, Presymptomatic detection of prions in blood. *Science* **313**, 92–94 (2006).
24. F. Moda, P. Gambetti, S. Notari, L. Concha-Marambio, M. Catania, K.-W. Park, E. Maderna, S. Suardi, S. Haïk, J.-P. Brandel, J. Ironside, R. Knight, F. Tagliavini, C. Soto, Prions in the urine of patients with variant Creutzfeldt–Jakob disease. *N. Engl. J. Med.* **371**, 530–539 (2014).
25. European Commission, *Technical specifications for in vitro diagnostic medical devices* (Official Journal of the European Union, 2011).
26. G. P. Saborio, B. Permanne, C. Soto, Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* **411**, 810–813 (2001).
27. M. B. Fischer, C. Roeckl, P. Parizek, H. P. Schwarz, A. Aguzzi, Binding of disease-associated prion protein to plasminogen. *Nature* **408**, 479–483 (2000).
28. M. Maissen, C. Roeckl, M. Glatzel, W. Goldmann, A. Aguzzi, Plasminogen binds to disease-associated prion protein of multiple species. *Lancet* **357**, 2026–2028 (2001).
29. C. E. Mays, C. Ryou, Plasminogen stimulates propagation of protease-resistant prion protein in vitro. *FASEB J.* **24**, 5102–5112 (2010).
30. C. Segarra, D. Bougard, M. Moudjou, H. Laude, V. Béringue, J. Coste, Plasminogen-based capture combined with amplification technology for the detection of PrP^{TSE} in the pre-clinical phase of infection. *PLOS ONE* **8**, e69632 (2013).
31. L. Concha-Marambio, S. Pritzkow, F. Moda, F. Tagliavini, J. Ironside, P. Schulz, C. Soto, Detection of prions in blood from patients with variant Creutzfeldt-Jakob disease. *Sci. Transl. Med.* **8**, 370ra183 (2016).
32. J. Y. Douet, S. Zafar, A. Perret-Liaudet, C. Lacroux, S. Lugan, N. Aron, H. Cassard, C. Ponto, F. Corbière, J. M. Torres, I. Zerr, O. Andreoletti, Detection of infectivity in blood of persons with variant and sporadic Creutzfeldt-Jakob disease. *Emerg. Infect. Dis.* **20**, 114–117 (2014).
33. R. Atarashi, J. M. Wilham, L. Christensen, A. G. Hughson, R. A. Moore, L. M. Johnson, H. A. Onwubiko, S. A. Priola, B. Caughey, Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. *Nat. Methods* **5**, 211–212 (2008).
34. J. M. Wilham, C. D. Orrú, R. A. Bessen, R. Atarashi, K. Sano, B. Race, K. D. Meade-White, L. M. Taubner, A. Timmes, B. Caughey, Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. *PLOS Pathog.* **6**, e1001217 (2010).
35. R. Atarashi, K. Satoh, K. Sano, T. Fuse, N. Yamaguchi, D. Ishibashi, T. Matsubara, T. Nakagaki, H. Yamanaka, S. Shirabe, M. Yamada, H. Mizusawa, T. Kitamoto, G. Klug, A. McGlade, S. J. Collins, N. Nishida, Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. *Nat. Med.* **17**, 175–178 (2011).
36. L. I. McGuire, A. H. Peden, C. D. Orrú, J. M. Wilham, N. E. Appleford, G. Mallinson, M. Andrews, M. W. Head, B. Caughey, R. G. Will, R. S. G. Knight, A. J. E. Green, Real time quaking-induced conversion analysis of cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease. *Ann. Neurol.* **72**, 278–285 (2012).
37. C. D. Orrú, M. Bongianni, G. Tonoli, S. Ferrari, A. G. Hughson, B. R. Groveman, M. Fiorini, M. Pocchiari, S. Monaco, B. Caughey, G. Zanusso, A test for Creutzfeldt–Jakob disease using nasal brushings. *N. Engl. J. Med.* **371**, 519–529 (2014).
38. A. H. Peden, L. I. McGuire, N. E. J. Appleford, G. Mallinson, J. M. Wilham, C. D. Orrú, B. Caughey, J. W. Ironside, R. S. Knight, R. G. Will, A. J. E. Green, M. W. Head, Sensitive and specific detection of sporadic Creutzfeldt–Jakob disease brain prion protein using real-time quaking-induced conversion. *J. Gen. Virol.* **93**, 438–449 (2012).
39. M. Gillies, G. Chohan, C. A. Llewelyn, J. MacKenzie, H. J. T. Ward, P. E. Hewitt, R. G. Will, A retrospective case note review of deceased recipients of vCJD-implicated blood transfusions. *Vox Sang.* **97**, 211–218 (2009).
40. S. Mead, J. D. F. Wadsworth, M.-C. Porter, J. M. Linehan, W. Pietkiewicz, G. S. Jackson, S. Brandner, J. Collinge, Variant Creutzfeldt-Jakob disease with extremely low lymphoreticular deposition of prion protein. *JAMA Neurol.* **71**, 340–343 (2014).
41. B. Chen, R. Morales, M. A. Barria, C. Soto, Estimating prion concentration in fluids and tissues by quantitative PMCA. *Nat. Methods* **7**, 519–520 (2010).
42. S. el Tawil, G. Mackay, L. Davidson, D. Summers, R. Knight, R. Will, Variant Creutzfeldt-Jakob disease in older patients. *J. Neurol. Neurosurg. Psychiatry* **86**, 1279–1280 (2015).
43. J. P. Brandel, A. Welaratne, D. Salomon, I. Capek, V. Vaillant, A. Aouba, S. Haïk, A. Alpérovitch, Can mortality data provide reliable indicators for Creutzfeldt-Jakob disease surveillance? A study in France from 2000 to 2008. *Neuroepidemiology* **37**, 188–192 (2011).
44. M. Zeildler, C. Gibbs, F. Meslin, WHO manual for strenthening diagnosis and surveillance of Creutzfeldt-Jakob disease. (1998).
45. I. Zerr, K. Kallenberg, D. M. Summers, C. Romero, A. Taratuto, U. Heinemann, M. Breithaupt, D. Varges, B. Meissner, A. Ladogana, M. Schuur, S. Haik, S. J. Collins, G. H. Jansen, G. B. Stokin, J. Pimentel, E. Hewer, D. Collie, P. Smith, H. Roberts, J. P. Brandel, C. van Duijn, M. Pocchiari, C. Begue, P. Cras, R. G. Will, P. Sanchez-Juan, Updated clinical diagnostic criteria for sporadic Creutzfeldt-Jakob disease. *Brain* **132**, 2659–2668 (2009).
46. V. Béringue, A. Le Dur, P. Tixador, F. Reine, L. Lepourry, A. Perret-Liaudet, S. Haïk, J.-L. Vilotte, M. Fontés, H. Laude, Prominent and persistent extraneural infection in human PrP transgenic mice infected with variant CJD. *PLOS ONE* **3**, e1419 (2008).
47. C. Segarra, S. Lehmann, J. Coste, Prion protein expression and processing in human mononuclear cells: The impact of the codon 129 prion gene polymorphism. *PLOS ONE* **4**, e5796 (2009).
48. C. J. Clopper, E. S. Pearson, The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika* **26**, 404–413 (1934).
49. R. C. Team, R: A language and environment for statistical computing. (2014).
50. K. Peoc'h, P. Manivet, P. Beaudry, F. Attane, G. Besson, D. Hannequin, N. Delasnerie-Laupêtre, J.-L. Laplanche, Identification of three novel mutations (E196K, V203I, E211Q) in the prion protein gene (*PRNP*) in inherited prion diseases with Creutzfeldt-Jakob disease phenotype. *Hum. Mutat.* **15**, 482 (2000).

Acknowledgments: We thank the Etablissement Français du Sang including L. Bruyère-Ostells for the helpful technical assistance and N. Coudurier and F. Roubinet for the preservation of preclinical samples. We thank C. Soto (University of Texas Medical School, Houston) for hosting us in his laboratory and for his advice about PMCA, P. Minor (NIBSC, U.K.) and A. Perret-Liaudet (CHU-Lyon, France) for providing us with sCJD brain samples, and L. Peckeu from the French Epidemiological Network Surveillance. This work was performed in the L3 facility [Etablissement Confiné d'Expérimentation–Centre d'Elevage et de Conditionnement Expérimental des Modèles Animaux (ECE-CECEMA)] of the University of Montpellier, France. The manuscript has been revised for English by an independent scientific language editing service (AngloScribe, Calvisson, France). **Funding:** This work was largely supported by the Etablissement Français du Sang as well as by a grant from the Alliance Biosecure Foundation (Prion Blood Confirm valid project) to J.C., INSERM, the Institut de Veille Sanitaire, the program Investissements d'avenir ANR-10-IAIHU-06, and the U.K. Department of Health Policy Research Programme (PRST061400008). **Author contributions:** D.B. and J.C. designed the study. D.B., V.B., C.S., and J.C. assisted in the development of the methods and analyzed all the samples with M.B., C.M., and S.N. D.B. and J.C. analyzed the data with contributions from S.N., J.-P.B., P.T., and S.H. D.B., M.B., C.M., and S.N. prepared the figures. J.-P.B., H.F., J.-L.L., A.G., A.W., D.N., R.K., R.W., and S.H. investigated the patients and obtained, processed, and documented the clinical samples. D.B. and J.C. drafted the paper with contributions from J.-P.B., S.N., C.F.-W., R.W., P.T., and S.H. **Competing interests:** R.W. is a member of the scientific advisory boards of LFB and Ferring Pharmaceuticals. Etablissement Français du Sang and C.S., J.C., and D.B. hold patents (US 13/425 806, FR 1152298, and EP 12160615.6, 2011, "Nanobeads covered with plasminogen as a direct support for cyclic amplification of the prion protein PrPSc") related to this work. All other authors declare that they have no competing interests.

Submitted 10 October 2015
Resubmitted 12 May 2016
Accepted 17 November 2016
Published 21 December 2016
10.1126/scitranslmed.aag1257

Citation: D. Bougard, J.-P. Brandel, M. Bélondrade, V. Béringue, C. Segarra, H. Fleury, J.-L. Laplanche, C. Mayran, S. Nicot, A. Green, A. Welaratne, D. Narbey, C. Fournier-Wirth, R. Knight, R. Will, P. Tiberghien, S. Haïk, J. Coste, Detection of prions in the plasma of presymptomatic and symptomatic patients with variant Creutzfeldt-Jakob disease. *Sci. Transl. Med.* **8**, 370ra182 (2016).

Science Translational Medicine
AAAS

www.sciencetranslationalmedicine.org/cgi/content/full/8/370/370ra182/DC1

Supplementary Materials for

Detection of prions in the plasma of presymptomatic and symptomatic patients with variant Creutzfeldt-Jakob disease

Daisy Bougard,* Jean-Philippe Brandel, Maxime Bélondrade, Vincent Béringue, Christiane Segarra, Hervé Fleury, Jean-Louis Laplanche, Charly Mayran, Simon Nicot, Alison Green, Arlette Welaratne, David Narbey, Chantal Fournier-Wirth, Richard Knight, Robert Will, Pierre Tiberghien, Stéphane Haïk, Joliette Coste*

*Corresponding author. Email: joliette.coste@efs.sante.fr (J.C.); daisy.bougard@efs.sante.fr (D.B.)

Published 21 December 2016, *Sci. Transl. Med.* **8**, 370ra182 (2016)
DOI: 10.1126/scitranslmed.aag1257

This PDF file includes:

Supplementary Materials:

Methods

Codon 129 genotyping

Patients or their relatives gave written informed consent for the genetic study. Genomic DNA was extracted from peripheral blood leukocytes using a Macherey Nagel kit, according to the manufacturer's instructions. The coding sequence of PRNP was amplified and analyzed as described in Peoc'h et al (*50*).

Table S1. Codon 129 genotype of the patients included in this study (including presymptomatic cases).

	MM	**MV**	**VV**	Not tested	**Total**
vCJD	20	0	0	0	**20**
sCJD	42	12	13	0	**67**
Other neurological disorders	5	11	3	48	**67**
Total	**67**	**23**	**16**	**48**	**154**

感染症研究報告　調査報告書

調査報告書番号	KK-JBPO-K20180011	文献ID	
研究報告の題目	Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease and Variant Creutzfeldt-Jakob Disease by Blood and Blood Products; Guidance for Industry		

研究報告の概要	報告企業の意見
業界向けガイダンス「クロイツフェルト・ヤコブ病（CJD）および変異型クロイツフェルト・ヤコブ病（vCJD）の血液および血液製剤を介した伝播リスクを低減するための改訂予防対策」の改正案： FDAにより、CJD及びvCJDの血液及び血液製剤を介した伝播リスクを低減するための業界向けガイダンス（改訂版）の改正案が提案された。主な改正内容（渡航歴の献血延期基準）は以下の通り。 ①現在：1980年から現在までにフランスや他のヨーロッパ各国の滞在が累積5年以上 提案：1980～2001年までの間のフランスかアイルランドの滞在が累積5年以上 ②現在：1980年から現在までにUKとフランスにおける輸血歴 提案：1980年から現在までにUK、フランス、アイルランドにおける輸血歴	血漿分画製剤は理論的なvCJD伝播リスクを完全には排除できないため、投与の際には患者への説明が必要である旨を2003年5月から添付文書に記載している。2009年2月17日、英国健康保護庁(HPA)はvCJDに感染した供血者の血漿が含まれる原料から製造された第Ⅷ因子製剤の投与経験のある血友病患者一名から、vCJD異常プリオン蛋白が検出されたと発表したが、弊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、国際獣疫事務局（OIE）により、日本及び米国は「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、プリオン病とされるアルツハイマー病とパーキンソン病については輸血を介して感染しないことが示唆されている。CJDは種類によって感染性が異なることが知られており、脳・脊髄、網膜などに異常型プリオン蛋白質が蓄積されるsCJDは、vCJDとは異なり、血液を介した感染を示す報告はこれまでのところないが、その潜在的可能性を示唆する報告もあるため、今後もCJDの情報については注意深く情報の収集、監視に努める。

今後の対応	その他参考事項等
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。	代表として献血ヴェノグロブリンIH5%静注0.5g/10mL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。

（注意）
1 複数の調査報告書を作成する場合は、シートをコピーして入力すること。

Amendment to "Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease and Variant Creutzfeldt-Jakob Disease by Blood and Blood Products; Guidance for Industry"

Draft Guidance for Industry

This guidance document is for comment purposes only.

Submit one set of either electronic or written comments on this draft guidance by the date provided in the *Federal Register* notice announcing the availability of the draft guidance. Submit electronic comments to https://www.regulations.gov. Submit written comments to the Dockets Management Staff (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Rm. 1061, Rockville, MD 20852. You should identify all comments with the docket number listed in the notice of availability that publishes in the *Federal Register*.

Additional copies of this guidance are available from the Office of Communication, Outreach and Development (OCOD), 10903 New Hampshire Ave., Bldg. 71, Rm. 3128, Silver Spring, MD 20993-0002, or by calling 1-800-835-4709 or 240-402-8010, or email ocod@fda.hhs.gov, or from the Internet at https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.

For questions on the content of this guidance, contact OCOD at the phone numbers or email address listed above.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
December 2017

Table of Contents

Contains Nonbinding Recommendations

Draft – Not for Implementation

Amendment to "Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease and Variant Creutzfeldt-Jakob Disease by Blood and Blood Products; Guidance for Industry"

Draft Guidance for Industry

This draft guidance, when finalized, will represent the current thinking of the Food and Drug Administration (FDA or Agency) on this topic. It does not establish any rights for any person and is not binding on FDA or the public. You can use an alternative approach if it satisfies the requirements of the applicable statutes and regulations. To discuss an alternative approach, contact the FDA staff responsible for this guidance as listed on the title page.

I. INTRODUCTION

This guidance, when finalized, will amend the document entitled "Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease and Variant Creutzfeldt-Jakob Disease by Blood and Blood Products; Guidance for Industry" updated January 2016 (the "2016 vCJD Guidance") (Ref. 1). The guidance provides revised recommendations intended to reduce the possible risk of transmission of variant Creutzfeldt-Jakob Disease (vCJD) by blood and blood products by: (1) revising and removing certain recommended deferrals for geographic risk of bovine spongiform encephalopathy (BSE) exposure; and (2) recommending deferral for individuals with a history of blood transfusion in Ireland from 1980 to the present.

The recommendations in this guidance apply to the collection of Whole Blood and blood components intended for transfusion or for use in further manufacturing into injectable and non-injectable products, including recovered plasma, Source Leukocytes and Source Plasma. Within this document, "donors" refers to donors of Whole Blood and blood components and "you" refers to blood collection establishments.

When this draft guidance is finalized, we, FDA, will amend the 2016 vCJD Guidance by incorporating into an updated final guidance any new recommendation adopted. All other recommendations in the 2016 vCJD Guidance will remain unchanged.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

A. CJD and vCJD

Creutzfeldt-Jakob Disease (CJD) is a rare but invariably fatal degenerative disease of the central nervous system, one of a group of transmissible diseases called transmissible spongiform encephalopathies (TSEs) or prion diseases. TSEs are associated with poorly understood transmissible agents (Refs. 2-7), now designated TSE agents or prions (Ref. 8). Cases of sporadic CJD—the most common human TSE—occur at low frequency by an unknown mechanism. CJD may be acquired by an identified exogenous exposure (usually iatrogenic) to infectious material, or it may be familial, associated with one of a number of mutations in the prion-protein-encoding (*PRNP*) gene. Clinical latency for iatrogenic CJD, following point exposures to contaminated materials, has sometimes exceeded 30 years (Ref. 9); incubation periods for kuru—another human TSE—have sometimes exceeded 50 years (Ref. 10).

In 1996, a previously unrecognized variant of CJD, now designated variant CJD (vCJD), was reported in the United Kingdom (U.K.) (Ref. 11). vCJD is distinguished from CJD by differences in clinical presentation, cerebral imaging, neuropathologic changes, and other features (Refs. 11-15). Laboratory and epidemiologic studies have linked vCJD to human infection with the agent of BSE, probably acquired from contaminated beef products (Refs. 16, 17). BSE was first recognized in the U.K. in 1985 and spread to most European countries and beyond (Ref. 18). The BSE and vCJD epidemics are currently in decline, although BSE has not been eradicated (Refs. 18, 19).

B. vCJD Risk in Blood

Early studies with blood of experimentally TSE-infected animals suggested that blood contained very low levels of infectivity (orders of magnitude less than in brain) but often sufficient to transmit the disease to susceptible animals. These results and the unique accumulation of abnormal prion protein seen in lymphoid tissues of persons with vCJD (but not in other forms of CJD) led to concerns that transmission of vCJD by blood might pose a greater risk than for sporadic CJD (Ref. 20). U.K. authorities have reported four transmissions of vCJD infection (three overt, one latent) by transfusions of non-leukocyte reduced red blood cells (RBC) and one possible transmission of vCJD by plasma-derived Factor VIII (Refs. 21-24). vCJD infectivity is present in the blood of affected individuals during the asymptomatic phase of disease for at least 3.5 years prior to onset of overt illness. In the U.K., donors unknowingly infected with vCJD and healthy at the time of

donation donated blood that transmitted vCJD to some recipients. These cases in the U.K. provided convincing epidemiological evidence that human blood carries the infectious vCJD agent and that the disease is transmissible by blood transfusion and plasma products (as it is with blood of TSE-infected animals). No cases of transfusion-transmitted vCJD (TTvCJD) have been reported in recipients of leukocyte reduced RBC. No cases of TTvCJD have been reported in the United States (U.S).

C. FDA Rationale for Revised Donor Deferral Recommendations for Geographic Risk of BSE Exposure

Starting in 1999, FDA issued several guidance documents intended to reduce the risk of TTvCJD by recommending that blood establishments defer donors who had spent time in certain countries where the risk of dietary exposure to the BSE agent was higher than that in the U.S. (Ref. 1). The deferral policy is likely to have reduced the risk of TTvCJD (Ref. 25). However, the deferrals have also eliminated a substantial number of otherwise eligible blood donors, most of whom are unlikely to be infected with vCJD. Based on these considerations and on the likely beneficial effect of leukocyte reduction in preventing about 54% of TTvCJD (Refs. 26-28) and acknowledging a marked decline of the BSE and vCJD epidemics worldwide (Refs. 18, 19), FDA decided to review the currently recommended geographic deferral policies.

On June 1, 2015, the FDA Transmissible Spongiform Encephalopathies Advisory Committee (TSEAC) discussed FDA's quantitative risk assessment estimating the effects of geographic donor deferrals in reducing the risk of TTvCJD. Based on results of the risk assessment, FDA is recommending a revised vCJD-related geographic deferral policy with consequent changes in the donor history questionnaire. A comparison of residual TTvCJD risk associated with current and modified deferral policies has been published (Ref. 25) and forms the basis of this draft amendment to the 2016 vCJD Guidance.

1. Risk Assessment

FDA developed a quantitative risk assessment based on a global geographic "risk ranking" model (Ref. 25) that estimated the contributions of donors potentially exposed to the BSE agent in various countries to total U.S. TTvCJD risk. The risk of exposure to the BSE agent was estimated either from the observed ("attributed") vCJD case rate of a country or from a rate "imputed" from probable exposure of the population to the BSE agent in beef products. The model then estimated potential person-years of potential BSE exposure by U.S. donors in the country (U.S. travelers visiting the country and immigrants to the U.S. from the country). FDA next used the model to evaluate both risk reduction and donor loss resulting from the current donor deferral policy compared with an alternative deferral option. FDA also evaluated a potential additional reduction in risk afforded by leukocyte reduction of RBC.

The model estimated that current geographic donor deferrals for vCJD risk combined with leukocyte reduction of RBC voluntarily implemented by blood establishments have reduced risk of vCJD transmission via RBC transfusion by approximately 90%. The model also indicated that U.K., Ireland, and France, the three countries with the highest vCJD risks, contributed 95% of the total TTvCJD risk in the U.S. Thus, by deferring donors only for time spent in these three countries and incorporating an assumption that 95% of all RBC currently transfused in the U.S. are leukocyte reduced (Ref. 29), the model predicted that the modified policy would maintain a level of blood safety similar to that resulting from current policy. A similar estimate for reducing risk of TTvCJD is presented in Table 1 in section II.C.3 of this guidance, on the alternative assumption that only 71.3% of RBC units are leukocyte reduced, consistent with a recent national survey report (Ref. 30). Based on these results, FDA is proposing to recommend deferral only for donors who spent time in U.K., Ireland, and France (and donors exposed to U.K. beef on certain U.S. military bases in Europe) and no longer recommending deferrals for time spent in all other European countries. These revisions would simplify the donor screening process and potentially allow more donors to donate. The other CJD-related and vCJD-related recommended deferrals in the 2016 vCJD Guidance would remain unchanged.

2. Leukocyte Reduction of Cellular Blood Components

Experience in the U.K. with universal leukocyte reduction of cellular blood components during the past 17 years has been far more encouraging than animal studies would have predicted (Refs. 27, 31). All four TTvCJD infections reported in the U.K. to date have been among a cohort of 27 persons transfused with non-leukocyte reduced RBC from donors who later developed symptomatic vCJD, while none of 25 transfusions of leukocyte reduced RBC from asymptomatic vCJD-infected donors have transmitted vCJD to recipients (Ref. 32). These compelling data indicate that leukocyte reduction reduces the risk of TTvCJD.

In addition to reducing the risk of TTvCJD, leukocyte reduction also provides other medical benefits. Leukocyte reduction is proven to reduce adverse effects attributed to leukocytes in transfused blood components including non-hemolytic febrile transfusion reactions, HLA alloimmunization and transmission of certain cell-associated blood-borne pathogens (e.g., cytomegalovirus, human T-cell lymphotropic viruses) (Refs. 33-36).

3. Risk Assessment Results

Table 1 provides the results from the risk assessment model. The FDA risk assessment model estimated the probable contribution of leukocyte reduction to lowering TTvCJD risk. Currently U.S. blood establishments voluntarily leukocyte reduce approximately 71.3-95% of transfused RBC units (Refs. 29, 30). Additional reduction in TTvCJD risk might be achieved if all RBC products were leukocyte reduced. FDA estimated the additional decreased risk from leukocyte reduction and

total risk reduction from combined donor deferral and leukocyte reduction. The estimated risk reduction by both the current 71.3-95% leukocyte reduction and universal leukocyte reduction of RBC, should that be implemented, were compared (see Table 1). Similar estimated reductions in total vCJD risk of close to 90% were achieved by both the current donor deferral policy and the proposed policy when either 71.3%, 95% or 100% of RBC were leukocyte reduced.

Table 1. Results from Risk Assessment Model

***Donor Deferral Strategy**		**Total percentage risk reduction (additional risk reduction)**			**Annual number of donors lost**
	Donor deferral only	**Donor deferral plus 71.3% RBC Leukocyte Reduction**	**Donor deferral plus 95% RBC Leukocyte Reduction**	**Donor deferral plus universal RBC Leukocyte Reduction**	
Model 1	79.0%	87.1% (8.1%)	89.8% (10.8%)	90.4% (0.6%)	254,091
Model 2	78.0%	86.5% (8.5%)	89.3% (11.3%)	89.9% (0.6%)	156,021

*****Model 1. Current donor deferral policy (U.K. >3 months, 1980-1996; other countries in Europe: >5 years, 1980-present).
*Model 2. Proposed donor deferral policy (U.K. >3 months, 1980-1996; France and Ireland: >5 years, 1980-2001).

Table 2 includes a summary of the proposed recommendations for geographical donor deferral changes. The proposed recommendations change the deferral for time spent in all European countries except for the U.K. Deferrals for time spent in the U.K. are unchanged because the risk assessment model results did not change the conclusions about U.K. risk of BSE exposure. Similarly, we are not changing the recommended deferral for individuals who spent time on military bases in Europe because their BSE exposure risk was from beef products sourced from the U.K. The risk assessment model also indicated that Ireland had a BSE risk similar to that of France. Therefore, we are recommending the same deferral period for time spent in France and Ireland and adding a deferral for individuals who had a blood transfusion in Ireland from 1980 to present.[1] The risk period for BSE exposure in Ireland and France is limited to 1980-2001 based on implementation of safeguards to the food chain by 2001 within the European countries (Ref. 37).

[1] Some unknown number of persons may remain latently infected with the vCJD agent long after dietary exposure to the BSE agent ended; it is not known if their blood would transmit infection to recipients. Until the situation becomes better understood, FDA recommends deferring anyone transfused in the U.K., Ireland, or France from 1980 to the present.

Table 2. Summary of Current Geographical vCJD Blood Donor Deferrals and the Proposed Deferrals

Current deferrals	**Proposed deferrals**
Donors who spent cumulatively ≥ 3 months in the U.K. from 1980 to 1996	Unchanged
Donors who spent cumulatively ≥ 5 years in France or other countries in Europe from 1980 to present	Donors who spent cumulatively ≥ 5 years in France or Ireland from 1980 to 2001
Donors with a history of blood transfusion in the U.K. and France from 1980 to the present	Donors with a history of blood transfusion in the U.K., France, or Ireland from 1980 to the present
Donors based on time and duration of exposure at military bases in Europe during periods in which commissaries and mess halls were supplied with beef products from the U.K.	Unchanged

4. 2015 TSEAC Meeting

FDA sought advice from TSEAC regarding revised geographic donor deferral policies to reduce the risk of TTvCJD. FDA presented results of the FDA risk assessment model predicting that deferral of donors who spent three months or longer in the U.K. from 1980 to the end of 1996 or five years or more in France or Ireland from 1980 through the end of 2001 plus leukocyte reduction of RBC (assumed to reduce risk of TTvCJD by about 54%) would maintain close to the current estimated level of risk reduction but allow a modest number of donors currently deferred to be reentered. FDA considered 2001 to be the year by which most European countries were to have implemented steps to protect food and animal feed from contamination with the BSE agent, steps similar to those adopted by 1996 in the U.K. Some TSEAC members disagreed with several assumptions used to develop the statistical model. FDA recognized the uncertainties of the risk assessment resulting from limitations of available information and agreed with the TSEAC members' concerns. Following the meeting, FDA investigated the issues raised by TSEAC and concluded that, although the TSEAC concerns were reasonable, none of the concerns changed the final results of the risk assessment or its final conclusion (Ref. 25).

At the meeting, the TSEAC also voted unanimously in favor of universal leukocyte reduction to reduce the risk of TTvCJD (Ref. 38).

III. RECOMMENDATIONS

The recommendations set forth below, when finalized, will update the donor deferral recommendations in the 2016 vCJD Guidance at sections IV.A.3-6, and 8 and IV.D.2.b. questions 1-4. All other recommendations in the guidance related to risk of CJD and familial TSEs will remain unchanged.

The following recommendations apply to the collection of Whole Blood and blood components intended for transfusion or for use in further manufacturing into injectable and non-injectable products, including recovered plasma, Source Leukocytes and Source Plasma.

A. Recommendations for Donor Deferral

We recommend that you defer donors for geographic risk of BSE exposure as follows:

1. Defer indefinitely a donor who has spent three months or more cumulatively in the U.K. from 1980 to 1996.

2. Defer indefinitely a donor who has spent five years or more cumulatively in France or Ireland[2] from 1980 to 2001.

3. Defer indefinitely former or current U.S. military personnel, civilian military personnel, and their dependents as follows:

    a. Individuals who resided at U.S. military bases in Northern Europe (Germany, U.K., Belgium, and the Netherlands) for six months or more from 1980 through 1990, or

    b. Individuals who resided at U.S. military bases elsewhere in Europe (Greece, Turkey, Spain, Portugal, and Italy) for six months or more from 1980 through 1996.

4. Defer indefinitely a donor with a history of blood transfusion in the U.K., France, or Ireland from 1980 to the present.

Appendix Table 1 in this guidance provides a summary of the current and revised recommendations for geographic risk of BSE exposure (see Appendix).

B. Recommendations for Donor History Questionnaire

We recommend that blood collection establishments update their donor history questionnaires (DHQ), including full-length and abbreviated DHQs and accompanying materials (e.g., flow charts) and processes to incorporate the recommendations provided in this guidance.

We recommend that the updated DHQ and accompanying materials include the following elements to assess donors for geographic risk of BSE exposure:

[2] Note that Northern Ireland is part of the U.K.

1. A history of travel or residence between 1980 through 1996 that adds up to three months or more in the U.K. (England, Northern Ireland, Scotland, Wales, the Isle of Man, the Channel Islands, Gibraltar, or the Falkland Islands).

2. A history of receiving a transfusion of blood, platelets, plasma, cryoprecipitate, or granulocytes since 1980 in the U.K. (England, Northern Ireland, Scotland, Wales, the Isle of Man, the Channel Islands, Gibraltar, or the Falkland Islands), Ireland, or France.

3. A history of serving as a member of the military, a civilian military employee, or a dependent of a member of the U.S. military between 1980 and 1996 and spending a total time of six months or more associated with a military base in any of the following countries:

    - From 1980 through 1990 in Belgium, the Netherlands, or Germany, or
    - From 1980 through 1996 in Spain, Portugal, Turkey, Italy, or Greece.

4. A history of travel or residence that adds up to five years or more in France or Ireland from 1980 through 2001 (including time spent in the U.K. from 1980 through 1996).

Appendix Table 2 in this guidance provides a summary of the current and revised recommendations for DHQ (see Appendix).

C. Donor Requalification

Under 21 CFR 630.35, you may determine a deferred donor to be eligible if, at the time of the current collection, the criteria that were the basis for the previous deferral are no longer applicable. For donors deferred for reasons other than under 21 CFR 610.41(a), you must determine that the donor has met criteria for requalification by a method or process found acceptable for such purposes by FDA (21 CFR 630.35(b)).

Accordingly, donors who were previously deferred because they spent five years or more in France or other countries in Europe since 1980 may be eligible to donate provided that they would not be deferred under section III.A of this guidance and they meet all other donor eligibility criteria.

IV. IMPLEMENTATION

Note: This guidance is being issued for comment purposes only. Implementation of the recommendations contained herein is not recommended at this time.

When this guidance is finalized, you may implement any revised recommendations once you have revised your DHQs, including the full-length and abbreviated DHQs, and accompanying materials to reflect the new donor deferral recommendations.

Licensed blood establishments must report the revisions to FDA in the following manner (21 CFR 601.12):

1. Revision of your own DHQs and accompanying materials must be submitted to FDA as a prior approval supplement (PAS) under 21 CFR 601.12(b).

2. Revision of a previously FDA accepted DHQ and accompanying materials must be reported as a major change if you are revising the FDA accepted DHQ and accompanying materials to implement these new recommendations. Report such a change to FDA as a PAS under 21 CFR 601.12(b).

   We recommend that you include the following in the PAS submission:

   a. Form FDA 356h "Application to Market a New or Abbreviated New Drug or Biologic for Human Use" which may be obtained at https://www.fda.gov/AboutFDA/ReportsManualsForms/Forms/default.htm;

   b. A cover letter describing the request and the contents of the submission;

   c. The DHQ and accompanying document(s). Please highlight the modifications.

3. If the current version of the DHQs and accompanying materials prepared by the AABB Donor History Task Force or Plasma Protein Therapeutics Association are revised to contain the recommendations in this guidance and are found acceptable by FDA, we would consider the implementation of the DHQ and accompanying materials to be minor changes, if implemented without modification and in their entirety as a complete process for administering questions to donors. Report such a change to FDA in your annual report under 21 CFR 601.12(d), noting the date the process was implemented (see 21 CFR 601.12(a)(3)).

V. REFERENCES

1. FDA "Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease and Variant Creutzfeldt-Jakob Disease by Blood and Blood Products; Guidance for Industry" dated May 2010 and updated January 2016. https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM307137.pdf
2. DeArmond, S. J. and S. B. Prusiner (1995). "Etiology and pathogenesis of prion diseases." Am J Pathol **146**(4):85-811.
3. Masters, C. L., J. O. Harris, et al. (1979). "Creutzfeldt-Jakob disease: patterns of worldwide occurrence and the significance of familial and sporadic clustering." Ann Neurol **5**(2):177-188.
4. Chesebro, B. (1998). "BSE and Prions: Uncertainties about the agent." Science **279**(5347):42-43.
5. Erdtmann, R. and L. Sivitz, Eds. (2001). Advancing Prion Science. Guidance for the National Prion Research Program (Institute of Medicine, U.S. Committee on Transmissible Spongiform Encephalopathies. Assessment of Relevant Science). Washington, The National Academies Press.
6. Chesebro, B. (2003). "Introduction to the transmissible spongiform encephalopathies or prion diseases." Br Med Bull **66**:1-20.
7. Manuelidis, L., Z. X. Yu, et al. (2007). "Cells infected with scrapie and Creutzfeldt-Jakob disease agents produce intracellular 25-nm virus-like particles." Proc Natl Acad Sci USA **104**(6):1965-1970.
8. Prusiner, S. B. (1982). "Novel proteinaceous infectious particles cause scrapie." Science **216**:136-144.
9. Croes, E. A., G. Roks, et al. (2002). "Creutzfeldt-Jakob disease 38 years after diagnostic use of human growth hormone." J Neurol Neurosurg Psychiatry **72**(6):792-793.
10. Collinge, J., J. Whitfield, et al. (2006). "Kuru in the 21st century--an acquired human prion disease with very long incubation periods." Lancet **367**(9528):2068-2074.
11. Will, R. G., J. W. Ironside, et al. (1996). "A new variant of Creutzfeldt-Jakob disease in the UK." Lancet **347**(9006):921-925.
12. Cousens, S. N., E. Vynnycky, et al. (1997). "Predicting the CJD epidemic in humans." Nature **385**(6613):197-198.
13. Zeidler, M., E. C. Johnstone, et al. (1997). "New variant Creutzfeldt-Jakob disease: psychiatric features." Lancet **350**(9082):908-910.
14. Zeidler, M., G. E. Stewart, et al. (1997). "New variant Creutzfeldt-Jakob disease: neurological features and diagnostic tests." Lancet. **350**(9082):903-907.
15. Ironside, J. W. (1998). "Neuropathological findings in new variant CJD and experimental transmission of BSE." FEMS Immunol Med Microbiol **21**(2):91-95.
16. Collinge, J., K. C. Sidle, et al. (1996). "Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD." Nature **383**(6602):685-690.
17. Bruce, M. E., R. G. Will, et al. (1997). "Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent." Nature **389**(6650):498-501.
18. World Organization for Animal Health (OIE). http://www.oie.int/animal-health-in-the-world/bse-specific-data/number-of-reported-cases-worldwide-excluding-the-united-kingdom/

19. Budka H., Will R.G. "The end of the BSE saga: do we still need surveillance for human prion diseases?" Swiss Med Wkly 2015; 145:w14212.
20. Will, R. G. and R. H. Kimberlin (1998). "Creutzfeldt-Jakob disease and the risk from blood or blood products." Vox Sanguinis (Basel)**75**(3):178-180.
21. Llewelyn, C. A., P. E. Hewitt, et al. (2004). "Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion." Lancet **363**(9407):417-421.
22. Peden, A. H., M. W. Head, et al. (2004). "Preclinical vCJD after blood transfusion in a *PRNP* codon 129 heterozygous patient." Lancet **364**(9433):527-529.
23. U.K. Health Protection Agency (2006). "New case of transfusion-associated vCJD." Commun Dis Resp CDR Wkly **16**:serial online.
24. Hewitt, P. E., C. A. Llewelyn, et al. (2006). "Creutzfeldt-Jakob disease and blood transfusion: results of the UK Transfusion Medicine Epidemiological Review study." Vox Sang **91**(3):221-230.
25. Yang H., Y. Huang, et al. (2017) "Geographic exposure risk of variant Creutzfeldt-Jakob disease in US blood donors: a risk-ranking model to evaluate alternative donor-deferral policies." Transfusion **57**(4):924-932.
26. Huang Y., L. Gregori, et al. (2014) "Development of dose-response models of Creutzfeldt-Jakob disease infection in nonhuman primates for assessing the risk of transfusion-transmitted variant Creutzfeldt-Jakob disease." J. Virol **88**:13732-13736.
27. Gregori L., N. McCombie, et al. (2004). "Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood." Lancet **364**(9433):529-531.
28. Gregori L., Yang H., Anderson S. (2011) "Estimation of variant Creutzfeldt-Jakob disease infectivity titers in human blood." Transfusion **51**(12):2596-2602.
29. Harvey A.R., S.V. Basavaraju, et al. (2015) "Transfusion-related adverse reactions reported to the National Healthcare Safety Network Hemovigilance Module, United States, 2010 to 2012." Transfusion **55**(4):709-718.
30. Ellingson K.D., M.R.P. Sapiano, et al. (2017) "Continued decline in blood collection and transfusion in the United States-2015." Transfusion **57 Suppl 2**:1588-1598.
31. McCutcheon S., A.R.A., et al. (2011) "All clinically-relevant blood components transmit prion disease following a single blood transfusion: a sheep model of vCJD." PLoS One **6**(8):e23169.
32. Transfusion Medicine Epidemiology Review (TMER). http://www.cjd.ed.ac.uk/projects/all-projects/transfusion-medicine-epidemiology-review-tmer
33. Heddle N.M., L. Klama, et al. (1994) "The role of the plasma from platelet concentrates in transfusion reactions." N. Engl. J. Med **331**(10):625-628.
34. King K.E., R.S. Shirey, et al. (2004) "Universal leukoreduction decreases the incidence of febrile nonhemolytic transfusion reactions to RBCs." Transfusion **44**:25-29.
35. Schlicter S.J. (1997) "Leukocyte reduction and ultraviolet b irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. The trial to reduce alloimmunization to platelets study group." N. Engl. J. Med **337**(26):1861-1869.
36. Bowden R.A. et al. (1995) "A comparison of filtered leukocyte-reduced and cytomegalovirus (CMV) seronegative blood products for the prevention of transfusion-associated CMV infection after marrow transplant." Blood **86**:3598-3603.

37. Regulation (EC) No 999/2001 of the European Parliament and of the Council, 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies
38. FDA Transmissible Spongiform Encephalopathies Advisory Committee. Transcript. June 1, 2015. http://wayback.archive-it.org/7993/20170406190928/https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/TransmissibleSpongiformEncephalopathiesAdvisoryCommittee/UCM455399.pdf.

APPENDIX

Appendix Table 1. Summary of Current and Revised Recommendations for Geographic Risk of BSE Exposure

Section of the 2016 vCJD Guidance	Current Recommendation	Proposed Recommendation
IV.A.	Donor deferral criteria 1-7 apply to all donors. Donor deferral criterion 8 (residence in Europe for 5 years or more between 1980 and the present) applies to all donors *with the exception of* donors of Source Plasma.	Donor deferral criteria 1-7 apply to all donors. Donor deferral criterion 8 is deleted.
IV.A.3	You should indefinitely defer donors who have spent 3 months or more cumulatively in the U.K. from the beginning of 1980 through the end of 1996.	Unchanged
IV.A.4	You should indefinitely defer donors who have spent 5 years or more cumulatively in France from the beginning of 1980 to the present.	You should indefinitely defer donors who have spent 5 years or more cumulatively in France or Ireland (but not Northern Ireland, which is part of the U.K.) from 1980 through 2001.
IV.A.5	You should indefinitely defer former or current U.S. military personnel, civilian military personnel, and their dependents as follows: a. Individuals who resided at U.S. military bases in Northern Europe (Germany, U.K., Belgium, and the Netherlands) for 6 months or more from 1980 through 1990, or b. Individuals who resided at U.S. military bases elsewhere in Europe (Greece, Turkey, Spain, Portugal, and Italy) for 6 months or more from 1980 through 1996.	Unchanged
IV.A.6	You should indefinitely defer donors who have received a transfusion of blood or blood components in the U.K. or in France between the beginning of 1980 and the present.	You should indefinitely defer donors who have received a transfusion of blood or blood components in the U.K. or in France or <u>in Ireland</u> from the beginning of 1980 to the present.
IV.A.8	You should indefinitely defer donors of Whole Blood, blood components for transfusion, and Source Leukocytes, who have lived cumulatively for 5 years or more in Europe from the beginning of 1980 until the present. (Note this criterion includes time spent in the U.K. from 1980 through 1996 and time spent in France from 1980 to the present.) Unless otherwise unsuitable (for example, because they lived in the U.K. or France or on U.S. military bases for the periods of time noted previously), these donors remain eligible for Source Plasma donation.	Deleted

Appendix Table 2. Summary of Current and Revised Recommendations for DHQ

Section of the 2016 vCJD Guidance	Current Recommendation	Proposed Recommendation
IV.D.2	Since the beginning of 1980, have you ever lived in or traveled to Europe? a. If the donor answers "No", you need not take any further action b. If the donor answers "Yes", then ask the following questions;	Delete
IV.D.2. question 1	Between 1980 through 1996 did you spend time that adds up to 3 months or more in the U.K. (England, Northern Ireland, Scotland, Wales, the Isle of Man, the Channel Islands, Gibraltar, or the Falkland Islands)?	Unchanged*
IV.D.2. question 2	Since 1980 have you received a transfusion of blood, platelets, plasma, cryoprecipitate, or granulocytes in the U.K. (England, Northern Ireland, Scotland, Wales, the Isle of Man, the Channel Islands, Gibraltar, or the Falkland Islands) or in France?	Assess donors for a history of receiving a transfusion of blood, platelets, plasma, cryoprecipitate, or granulocytes since 1980 in the U.K. (England, Northern Ireland, Scotland, Wales, the Isle of Man, the Channel Islands, Gibraltar, or the Falkland Islands), <u>Ireland</u> or France.
IV.D.2. question 3	Between 1980 through 1996, were you a member of the military, a civilian military employee, or a dependent of a member of the U.S. military? If the donor answers "No," you need not take any further action. If the donor answers "Yes," ask the following question: Did you spend a total time of 6 months or more associated with a military base in any of the following countries: • From 1980 through 1990 in Belgium, the Netherlands, or Germany, or • From 1980 through 1996 in Spain, Portugal, Turkey, Italy, or Greece? 4) From 1980 to 2001, have you spent time that adds up to 5 years or more in France or in Ireland?	Unchanged*
IV.D.2. question 4	Since 1980, have you spent time that adds up to 5 years or more in France?	Assess donors for a history of travel or residence that adds up to 5 years or more in France or Ireland from 1980 through 2001 (including time spent in the U.K. from 1980 through 1996).
IV.D.2. question 4 (alternative)	Since 1980, have you spent time that adds up to 5 years or more in Europe (including time spent in the U.K. from 1980 through 1996)?	Deleted

* Note that Section III.B. of this guidance does not recommend specific questions for inclusion on the DHQ.

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。

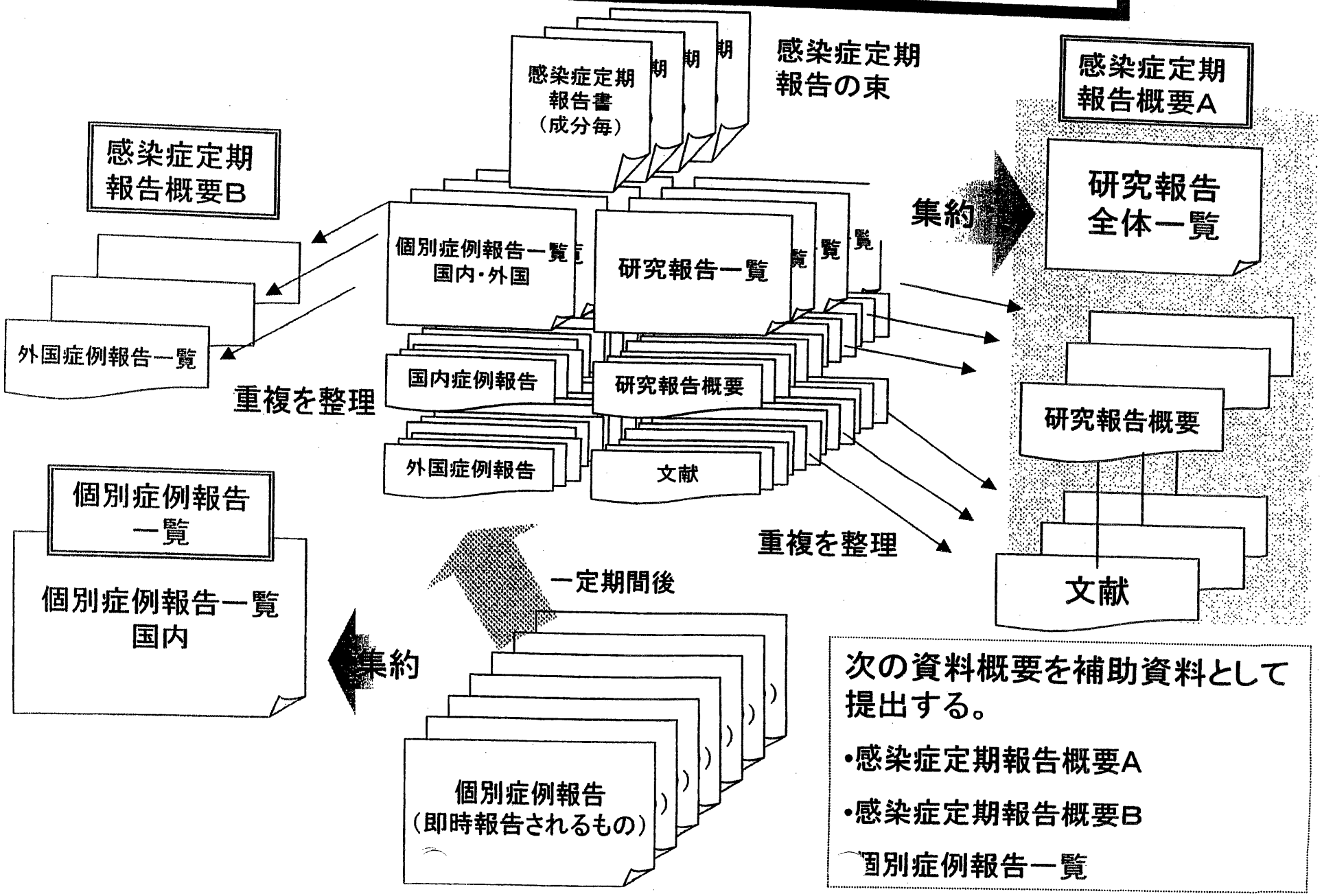
② 感染症発生症例報告のうち、発現国が「外国」の血漿分画製剤の使用による症例は、同一製品毎に報告期間を代表する感染症発生症例一覧（別紙様式第4）をまとめた**「資料概要B」**を事務局が作成し、送付する。

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