TABLE 2. Comparison of demographic, clinical, and laboratory findings in patients infected with the four dengue serotypes*

	PCR† type 1 (n=27)	PCR type 2 (n=13)	PCR type 3 (n=9)	PCR type 4 (n=7)	Overall (n=56)	P value
Gender (M:F)	13:14	6:7	5:4	3:4	27:29	1.0000
Age, median (IQR)	36.0 (24.0-52.0)	54.0 (33.0-66.0)	28.0 (23.5-61.0)	35.0 (21.0-63.0)	36.0 (26.3-57.8)	0.3559
Duration of hospitalisation, median (IQR) (days)	5.0 (4.0-7.0)	6.0 (3.0-7.5)	7.0 (4.5-8.5)	5.0 (4.0-6.0)	5.0 (4.0-7.0)	0.4589
Retro-orbital pain	9/18 (50)	3/10 (30)	0/4 (0)	1/6 (17)	13/38 (34)	0.2297
Rash—symptom	15/25 (60)	8/13 (62)	2/9 (22)	2/7 (29)	27/54 (50)	0.1332
Rash—sign	17/26 (65)	10/13 (77)	6/9 (67)	1/7 (14)	34/55 (62)	0.0509
Abdominal pain	3/25 (12)	2/12 (17)	1/8 (13)	1/7 (14)	7/52 (13)	1.0000
Diarrhoea	11/25 (44)	5/12 (42)	2/8 (25)	1/6 (17)	19/51 (37)	0.5956
Bleeding manifestation (epistaxis, gum bleeding, petechiae, haematuria)	13/26 (50)	8/12 (67)	5/8 (63)	2/6 (33)	28/52 (54)	0.5775
Hepatomegaly	2/26 (8)	2/13 (15)	0/9 (0)	1/7 (14)	5/55 (9)	0.5883
Leukopenia	25/26 (96)	10/13 (77)	9/9 (100)	5/7 (71)	49/55 (89)	0.0529
Lymphopenia	20/22 (91)	9/13 (69)	8/9 (89)	4/6 (67)	41/50 (82)	0.2550
Atypical lymphocyte	18/26 (69)	10/13 (77)	7/9 (78)	6/7 (86)	41/55 (75)	0.8848
Thrombocytopenia	26/26 (100)	11/13 (85)	8/9 (89)	6/7 (86)	51/55 (93)	0.0931
Elevated aspartate aminotransferase	8/9 (89)	3/4 (75)	4/4 (100)	2/2 (100)	17/19 (89)	1.0000
Elevated alanine aminotransferase	23/26 (88)	11/13 (85)	7/9 (78)	6/7 (86)	47/55 (85)	0.8954
Hypoalbuminaemia	10/26 (38)	5/13 (38)	5/9 (56)	4/7 (57)	24/55 (44)	0.6658
Highest temperature, mean (SD)	38.6 (1.0)	38.2 (1.1)	38.6 (1.3)	38.7 (0.6)	38.5 (1.0)	0.6893
Transfusion	4/23 (17)	2/12 (17)	1/8 (13)	2/6 (33)	9/49 (18)	0.8548

* Data are shown in No. (%), except otherwise stated
† PCR denotes polymerase chain reaction

TABLE 3. Demographic, clinical, and laboratory findings in patients with dengue haemorrhagic fever

Sex/age (years)	Ethnicity	Fever	Haemorrhagic manifestations	Lowest platelet count (x 10^9/L)	Plasma leakage	Laboratory findings	
						Serotype	Serology titer
M/38	Thai	37.2°C	Petechiae, bloody diarrhoea	9	Pleural effusion	Not done	Immunoglobulin M +ve
M/46	Chinese	38.4°C	Petechiae, bruises	9	Ascites	DEN 2	Immunoglobulin M +ve
F/49	Thai	38°C	Coffee ground vomitus, petechiae	8	Hypoalbuminaemia; haemoconcentration	DEN 1	4-fold increase*

* 1st titre: 640 (DEN-1), 5120 (DEN-2), 1280 (DEN-3), 1280 (DEN-4); 2nd titre: 5120 (DEN-1), 10 240 (DEN-2), 10 240 (DEN-3), 10 240 (DEN-4)

macrocytic anaemia and pancytopenia. She was diagnosed to have vitamin B12 deficiency anaemia, which was treated by vitamin B12 replacement and received a blood transfusion on 24 August 2002. On day 2 post-transfusion, she developed low-grade fever, but no skin rash, headache, myalgia, arthralgia, or retro-orbital pain. The patient was treated with antibiotics as for a urinary tract infection, based on the microbiological findings. The fever subsided 3 days later and the patient recovered uneventfully. The blood product she received was donated by a 17-year-old asymptomatic patient living in Ma Wan, during his viremic phase on 17 July 2002. On 24 July 2002, he developed generalised skin rash and attended the Accident and Emergency Department of Yan Chai Hospital. In October, he was subsequently picked up as one of the dengue cases based on serology results during the active case finding exercise in Ma Wan. Molecular testing performed on the donated blood product was positive for dengue virus type 1. The woman who had received the blood transfusion was recalled for blood testing on 7 October 2002, and was found to be positive for corresponding IgM antibodies and had a haemagglutination-inhibition titre of 1:2560. This incident was the first documented cases of such transmission in the literature, and since October

2002, the Hong Kong Red Cross Blood Transfusion Service (BTS) has intensified its donor deferral systems to counter this possibility. Specifically, it now asks about symptoms of dengue fever in the Blood Donor Registration Form (Supplement) by reminding all prospective donors to inform the BTS staff of all instances for flu, fever, headache, eye pain, muscle/joint pain, vomiting, and skin rash experienced 2 weeks before or after blood donation.

In our study, dengue fever was far more common than DHF and dengue shock syndrome, which were rare events. Our patients only manifested mild bleeding with good clinical outcomes and no fatalities. The clinical presentations of dengue fever, such as fever, myalgia, headache, and arthralgia, were comparable to findings reported in other studies.[10-12] Our patients (35%) presented with fewer gastroenteritis symptoms compared to those of others (50-98%).[11,12] Lymphadenopathy was documented in only 16% of our patients, which is much lower than the figure of 50% reported elsewhere.[13] This difference may be accounted for by less-than-adequate physical examination. Gum bleeding and epistaxis were reported in 12% and 10% of our patients respectively, which was also much lower than that reported previously.[11,12] Such differences could be due to the populations studied; patients recruited in endemic countries were mainly encountered during outbreaks in which both dengue fever and DHF were common. Previous studies showed dengue disease severity correlated with high viremia titres, secondary infection, and DEN-2 serotype infection.[14,15] Our findings showed that the haemorrhagic tendencies and duration of hospitalisation were not related to specific serotypes. Although some of our patients did receive platelet transfusions, the efficacy of such treatment in speeding recovery remains controversial. According to Thai experts, platelets are almost immediately destroyed by immune lysis after administration.[16]

Our study had several limitations. First, the target patients were limited to those with laboratory-confirmed dengue admitted to public hospitals. During 1998 to 2005, DH received notification of 203 dengue cases, including 77 who were admitted to private hospitals or consulted general practitioners only. The disease burden might also be underestimated, because some patients might have recovered, without seeking medical attention, while others might not have undergone serological testing. Second, statistical analysis could not be carried out to compare clinical and laboratory parameters in patients with dengue fever and DHF, as there were too few of the latter. Third, laboratory results before 2002 were not available in the Public Health Laboratory Information System. Fourth, not all clinical symptoms and signs listed in Table 2 could be retrieved from the medical records, as some may not have been specifically asked for or looked for.

In conclusion, dengue fever should be considered in the differential diagnosis of febrile patients with or without a travel history. Health care providers should therefore have an understanding of the infection, the spectrum of its clinical features, and methods of diagnosis and appropriate treatment. Until the *Aedes* mosquito can be effectively controlled or a cost-effective vaccine is developed, dengue fever will remain a public health concern, especially in South-East Asia. Control at source is one of the keys to combating dengue fever and requires active participation from all sectors of the community.

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医薬品　研究報告　調査報告書

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一般的名称	フィブリノゲン加第 XIII 因子		研究報告の公表状況	Clearance of dengue virus in the plasma-derived therapeutic proteins. Transfusion. 2008 Jul;48(7):1342-7. 2008 Feb 22.		公表国 中国	
販売名（企業名）	ベリプラスト P コンビセット （CSL ベーリング株式会社）						

研究報告の概要

問題点（血漿分画製剤でのデングウイルスの不活化・除去）

デングウイルスは年間に世界で 5 千万から 1 億人が感染し、感染者の数十万人がより重篤で生命を脅かすデング出血熱やデングショック症候群に進展する。

デングウイルスはフラビウイルス科に属し、直径 50nm のエンベロープを有する RNA ウイルスである。一般に血液などの高蛋白な体液で長期間生存するので、輸血により感染する可能性がある。針刺し事故や骨髄移植、分娩での血液に関連するデングウイルス感染が報告されている。

本研究は血漿分画製剤でのデングウイルス伝播の危険性が、特定のウイルス除去・不活化工程で除去されることを初めて証明するため実施された。

低温エタノール分画、陰イオン交換クロマトグラフィー、パスツリゼーション、S/D 処理とウイルスろ過を含むアルブミンやグロブリンの各製造工程前に、高力価の培養デングウイルスセロタイプ 2 を正常人血漿にスパイクし、各製造工程でのデングウイルスのクリアランスを $TCID_{50}$ アッセイ、RT-PCR で測定した。

デングウイルスの不活化・除去に対して、各製造工程前は全てで有効であった。

また、アルブミンの全製造工程（低温エタノール分画、パスツリゼーション）で少なくとも 10.12 log 減少すること、グロブリンの全製造工程（低温エタノール分画、ウイルスろ過、S/D 処理、クロマトグラフィー）では少なくとも 14.24 log 減少することが証明された。

現在実施されている血漿分画製剤の製造方法は、デングウイルス伝播に関して安全である。

使用上の注意記載状況・その他参考事項等

報告企業の意見	今後の対応
本剤の製造工程（低温エタノール分画、パスツリゼーション、イオン交換樹脂等）でデングウイルスが不活化・除去できると考えられる。	今後とも新しい感染症に関する情報収集に努める所存である。

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Clearance of dengue virus in the plasma-derived therapeutic proteins

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BACKGROUND: Viral safety is of paramount importance for human plasma–derived therapeutic proteins. Recent reports of blood-associated transmission and continuous regional outbreaks of dengue fever have prompted a validation of clearance of dengue virus in the manufacture processes of the plasma-derived products.

STUDY DESIGN AND METHODS: A high titer of cultured dengue virus serotype 2 was spiked into process samples before individual steps of albumin and immunoglobulin manufacture processes, including cold ethanol precipitation, cation-exchange chromatography, pasteurization, solvent/detergent treatment, and virus filtration. Clearance of dengue virus was quantified with $TCID_{50}$ assays in the culture of Vero E6 cells and, when appropriate, real-time polymerase chain reaction (RT-PCR) assays.

RESULTS: The individual process steps were all effective in the inactivation and/or removal of dengue virus, and the data obtained clearly demonstrate that the risk of dengue virus transmission was reduced cumulatively by at least 10.12 and at least 14.24 log in the albumin and immunoglobulin manufacture processes, respectively.

CONCLUSION: The dedicated viral inactivation and/or removal approaches currently implemented in the manufacture of plasma-derived products provide a good safety margin with regard to the transmission of dengue virus.

Dengue virus infects 50 to 100 million people worldwide a year; of those infected, several hundred thousand develop the more severe and life-threatening diseases, dengue hemorrhagic fever and dengue shock syndrome. Dengue virus belongs to the family Flaviviridae, which in general is known to survive over long periods in fluids with high protein contents, for example, blood. Therefore, dengue viruses may be transmitted via transfusion of blood or blood components. Albeit rare, it has indeed been documented that blood-associated transmission of dengue virus occurs via routes including needle-stick injuries,[1] marrow transplantation,[2] intrapartum and vertical transmission,[2] and mucocutaneous transmission.[3] This can be a serious public health problem without proper control measures.

Dengue virus is a lipid-enveloped RNA virus, with a diameter of approximately 50 nm.[4] Reportedly, dengue virus has been effectively inactivated by photosensitizers[5,6] and is sensitive to high temperatures and acidic pH.[7] This study aims to demonstrate for the first time that the

ABBREVIATION: BVDV = bovine viral diarrhea virus.

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risk of dengue virus transmission in plasma derivatives is eliminated by specific virus removal and inactivation procedures. Log reduction of dengue virus is investigated at individual steps of the manufacture processes of plasma-derived albumin and immunoglobulins, which include cold ethanol precipitation, cation-exchange chromatography, pasteurization, solvent/detergent (S/D) treatment, and virus filtration. The evaluation of the manufacture processes provides a measure of confidence for eliminating dengue virus.

MATERIALS AND METHODS

Raw materials

Normal human plasma was obtained from the plasma fractionator Shenzhen Weiwu Guangming Biological Products Co. (Shenzhen, China). All chemicals used in this study were of either pharmaceutical grade or analytical grade. Virus filters (Planova 35N, 10 cm^2) were a gift from Asahi Kasei (Tokyo, Japan).

Virus culture and quantification

Dengue virus serotype 2 (S047/00 from Environmental Health Institute, Singapore) was propagated in C6/36 cells (CDC Guangdong, China) in minimal essential medium with 1 percent fetal bovine serum (Gibco, Grand Island, NY). Dengue virus was quantified with $TCID_{50}$ assays in the culture of Vero E6 cells (ATCC, Manassas, VA). Vero E6 cells (2.5×10^5 cells/mL) were seeded in 96-well plates in a volume of 100 µL per well. After 1 day of incubation, 50 µL of medium was added to each well. Each dilution of sample was added at 50 µL per well, and further incubation was carried out at 36 ± 2°C with 5 percent CO_2. Plates were assessed for $TCID_{50}$ endpoint as cytopathic effects developed on the fifth day. The $TCID_{50}$ endpoint was calculated according to the Spearman-Kärber method, and the Poisson distribution was used when no virus was detected in samples. Quantitative real-time polymerase chain reaction (RT-PCR) was used to determine virus titer in the chromatography and cold ethanol precipitation steps. RNA of dengue virus was extracted in duplicate from samples with a viral RNA mini kit (QIAamp, Qiagen, Hilden, Germany) according to the procedure provided by the manufacturer. Dengue virus cDNA was reverse transcribed with random hexamers with reverse transcriptase (Supercript III, Invitrogen, Carlsbad, CA). Quantitative RT-PCR utilizing TaqMan technology (Applied Biosystems, Foster City, CA) was performed on samples and proper controls with specific primers (GTCAACATAGAAGCAGAACCTCCA and CTCTATGATGATGTAGCTGTCTCCG) and SYBR Green fluorescent probes with conditions optimized to detect 4.67 copies of viral RNA for dengue virus. Duplicate PCR procedures were performed for each sample with a sequence detection system (ABI 7900 HT, Applied Biosystems), and the cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute, as well as a dissociation stage of 95°C for 15 minutes, 60°C for 15 minutes, and 95°C for 15 minutes.

Fraction IV precipitation

The supernatant II + III was prepared from frozen human plasma through two consecutive steps of cold ethanol precipitation with 8 percent ethanol at pH 7.1 followed by 19 percent ethanol at pH 5.85.[8] Duplicates of 20 mL of supernatant II + III were spiked with 7.00 log per mL each of dengue virus at a ratio (vol/vol) of 1:10. Ethanol (95%) was added drop by drop into the supernatant II + III to a final ethanol concentration of 40 percent, which was further mixed at –5 to 5.5°C for 1 hour, before being centrifuged at 2300 × *g* to separate the fraction (F)IV from the supernatant IV. The supernatant II+III, the FIV, and the supernatant IV were titrated for quantity of viruses by $TCID_{50}$ assay or RT-PCR assay.

Pasteurization

The purified albumin solution was diafiltrated with 8 volumes of water and then concentrated to a concentration of 22 percent with a 30-kDa cutoff cassette (Millipore, Bedford, MA). Sodium caprylate was added to the concentrated albumin solution to a final concentration of 32 mmol per L, before adjustment of pH to 6.8 to make 20 percent albumin bulk. Two-hundred milliliters of albumin bulk and duplicates of the sterile-filtered 20 percent albumin in a 50-mL bottle was heated to 59°C in a water bath, followed by spiking with dengue virus (6.67, 7.50, or 7.67 log/mL) at a ratio (vol/vol) of 1:20 and 1:25, respectively. Gentle mixing with a mechanical stirrer (stainless steel) was applied to the bulk pasteurization. Samples were taken out for virus titration during the time course of a 10-hour treatment at 59 to 60°C.

FIII precipitation

The FII + III separated from the supernatant II + III above was redissolved, and NaAc-HAc buffer (0.8 mol/L-4 mol/L, pH 3.9) was added dropwise to adjust pH to 5.1. Dengue virus (7.17 or 7.67 log/mL) was spiked at a ratio (vol/vol) of 1:10 into duplicates of 20 mL of the pH-adjusted FII + III. Ethanol (95%) was added drop by drop into the FII + III to a final ethanol concentration of 15 percent, which was further mixed at –5 to 5.5°C for 1.5 hour, before being centrifuged at 2300 × *g* to separate the FIII from the supernatant III. The FII + III, the supernatant III, and the FIII were titrated for quantity of viruses by $TCID_{50}$ assay.

Virus filtration

A quantity of 196 mL of partially purified immunoglobulin was spiked with 7.67 log per mL dengue virus at a ratio (vol/vol) of 1:49, followed by filtration with a 0.22-µm filter (Steritop, Millipore) to remove viral aggregates. The filtered immunoglobulin was subject to virus filtration with the 35N filter in a normal-flow manner, under constant pressure of 80 kPa. Samples were titrated for quantity of viruses by $TCID_{50}$ assay or RT-PCR assay.

S/D treatment

Duplicates of 27 mL of the immunoglobulin purified through virus filtration were heated to 28°C in a water bath, followed by spiking with 7.16 or 7.83 log per mL dengue virus at a ratio (vol/vol) of 1:9. Triton X-100 and tri-*n*-butyl phosphate were added drop by drop into the immunoglobulin to a final concentration of 1 and 0.3 percent, respectively. Gentle mixing was achieved with a mechanical stirrer (stainless steel) for the time course of 16-hour treatment at 28 to 30°C, during which samples were removed for virus titration by $TCID_{50}$ assay.

Cation-exchange chromatography

A chromatography column of 10-mm diameter was packed to a bed height of 11 cm with either new CM Sepharose Fast Flow resin (Pharmacia Biotech, Uppsala, Sweden) or the used resin that had previously been recycled 476 times with the immunoglobulin purification process. The column was equilibrated with 20 mmol per L NaAc buffer, pH 4.0. Adjusted to a pH of 4.0 with 1 M HCl and an ionic strength of 1.4 mS per cm with purified water, duplicates of 75 mL of the S/D-treated immunoglobulin solution were spiked with 7.67 or 7.83 dengue virus at a ratio (vol/vol) of 1:20. The virus-spiked immunoglobulin solution was applied to the column at a linear flow rate of 40 cm/hr at ambient temperature. After washing of the column with 10 column volumes of 10 mmol per L glycine, pH 7.0, immunoglobulins were eluted with 100 mmol per L glycine together with 150 mmol per L sodium chloride, pH 9.0. The column load, the flow-through fraction, and the eluate fraction containing immunoglobulins were titrated for quantity of viruses by $TCID_{50}$ assay or RT-PCR assay.

RESULTS

FIV precipitation

After the 40 percent ethanol precipitation of the supernatant II + III, no dengue virus was detected with the $TCID_{50}$ assay in both the supernatant IV and the FIV (Table 1). Despite its direct cytotoxicity to the virus detector Vero E6 cells, when diluted 500-fold, 40 percent ethanol did not affect the determination of virus titer. Results of quantitative RT-PCR clearly showed that genetic materials of dengue virus were concentrated in the FIV (Table 1), which is discarded during the albumin manufacture. Because chemical inactivation by high concentrations of ethanol is mechanistically different from the physical partitioning effects between fractions, this FIV precipitation step provides an extra safety margin in the effective clearance of dengue viruses.

Pasteurization

The kinetics of inactivation of dengue virus in the 20 percent albumin during the 10-hour pasteurization at 59 to 60°C are shown in Fig. 1. The pasteurization was carried out at 0.5°C below what is normally used in the manufacture, representing a worst-case scenario. Dengue

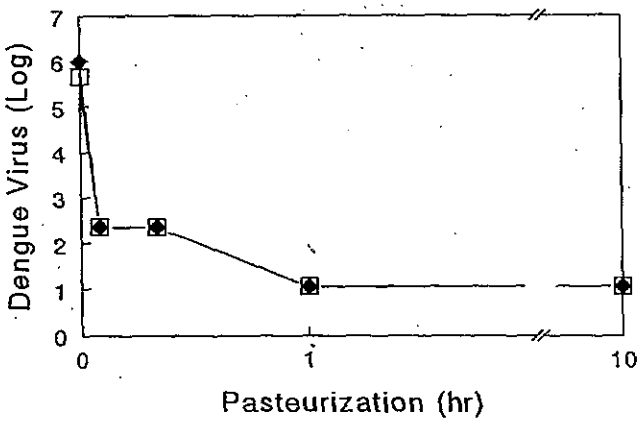

Fig. 1. Inactivation of dengue virus in albumin by pasteurization over time. (□) Bulk pasteurization; (◆) terminal pasteurization. Stock dengue viruses (6.67 or 7.50 log/mL) were spiked at a ratio (vol/vol) of 1:20 and 1:25, respectively, in the bulk pasteurization and terminal pasteurization.

TABLE 1. Clearance of dengue virus in the precipitation of FIV*

Assay (log)	Supernate II + III	FIV	Supernate IV	Log reduction, II + III → supernate IV
$TCID_{50}$	6.83/7.00	2.06†/2.06†	1.65†/1.65†	≥5.18/≥5.35
Quantitative RT-PCR	7.15/8.33	7.40/7.56	3.30/4.98	3.85/3.35

* Data shown are total viral titers (log number multiplied by volume) from duplicate experiments, where stock virus had a titer each of 7.00 log per mL spiked at a ratio of 1:10.
† No dengue virus was detected, and the number was calculated according to the Poisson distribution.