Table 1. Laboratory Data of Healthy Volunteers and Patients With Secondary Dengue Virus Infection During the Acute Phase

Diagnosis (No.)	Age (y), Median (IQR)	Days After Onset	Increase in Hematocrit, %	Platelet Count ($\times 10^3/\mu L$)	Thrombopoetin (pg/mL), Median (IQR)	Annexin V Binding, %	Active Caspase 3, %	Phagocytosis, %
Controls (38)	25 (22–28)	...	...	284.00 (65.36)	33.23 (20.52–40.46)	3.27 (0.91)	2.82 (1.05)	20.29 (5.77)
DV Infection (81)	23 (21–27)	3.69 (0.97)	17.19 (10.15)	45.52 (29.12)[a]	275.25 (109.8–391.96)[a]	19.67 (10.72)[a]	16.82 (11.31)[a]	55.46 (29.66)[a]
DF (57)	23 (20–27)	3.54 (0.95)	12.99 (7.08)	49.56 (29.71)	283.79 (111.33–385.44)	16.73 (10.43)	13.73 (10.43)	50.15 (28.67)
DHF (24)	24 (22–27)	4.04 (0.96)	27.18 (9.43)[b]	35.92 (25.74)[b]	216.50 (101.4–452.32)	25.72 (8.77)[b]	23.14 (10.61)[b]	68.09 (28.68)[b]

Data are expressed as the mean (SD) unless otherwise specified.

Abbreviations: DF, dengue fever; DHF, dengue hemorrhagic fever; DV, dengue virus; IQR, interquartile range.

[a] $P < .0001$ (vs controls);

[b] $P < .05$ (vs DF).

Platelet Apoptosis

Platelet apoptosis was examined in 58 patients and 28 controls. Platelets were prepared from the patients or controls, as described previously [8]. Purified platelets were stained with a platelet marker (phycoerythrin [PE]/Cy5-conjugated anti-CD41; BioLegend) and then stained with PE-conjugated annexin V (BD Pharmingen) for PS exposure. Platelets were also stained with FAM-DEVD-FMK (Immunochemistry Technologies) for active caspase 3 [13].

In Vitro Platelet Apoptosis and Phagocytosis of Apoptotic Platelets

DV1 (Mochizuki strain), DV2 (16681 strain), DV3 (H87 strain), DV4 (H241 strain), or Japanese encephalitis virus (JEV; Nakayama strain) was propagated in C6/36 mosquito cells in Leibovitz L-15 medium (Gibco) containing 2% fetal bovine serum and 0.29% tryptose phosphate broth. Human platelets isolated from healthy donors at a concentration of 10^6/mL were incubated in L-15 medium containing DV of each serotype or JEV at a multiplicity of infection of 1 or in medium only for 96 hours at 37°C. Platelets were harvested at the indicated time points and tested for annexin V binding. Platelets were pretreated with an unlabeled purified caspase 3 inhibitor (Z-DEVD-FMK [R&D Systems]) or with a caspase inhibitor negative control (CTR; Z-FA-FMK [R&D Systems]) at 1 μg/mL for 30 minutes at 37°C. Human platelets suspended in L-15 medium containing DV4 or in medium only were cultured for 96 hours and used for platelet phagocytosis. Platelets isolated from healthy donors were incubated in medium containing DV4 or in medium only for 72 hours; freshly isolated platelets were analyzed using Western blotting. Activation of caspase 3 was detected with a rabbit polyclonal antibody (Cell Signaling Technology) directed against procaspase 3 and its cleaved form. Detection of β-actin confirmed that equivalent amounts of protein were loaded.

Statistical Analysis

All data are shown as the mean (SD) for continuous variables with normal distribution or as the median (interquartile range) for those with nonnormal distribution. Platelet counts, platelet apoptosis, serum TPO levels, and platelet phagocytosis in patients during the acute, early convalescence, and convalescence phases and in controls were analyzed using repeated measures analysis of variance (ANOVA) and Scheffé method for the post hoc multiple comparisons test. Data from the assays of inhibition of platelet phagocytosis were analyzed using the independent samples t test. In vitro experiments aimed at examining the induction of apoptosis and platelet phagocytosis using healthy donor cells in different culture conditions were performed using ANOVA and Tukey multiple comparisons test. The significance of the correlations was estimated using the Pearson coefficient. $P < .05$ was considered significant. The SPSS statistical software version 16.0 (SPSS, an IBM Company) was used for all data analyses.

RESULTS

Patient Characteristics

Among the 81 patients, 57 were diagnosed with DF and 24 with DHF (Table 1). Patients with DHF were classified further into DHF grade I (n = 5) or grade II (n = 19). No fatal cases were included. The mean increase in hematocrit was significantly greater in DHF patients than in DF patients ($P < .0001$). The mean period from the onset of fever to the admission of the patients was 3.7 (1.0) days.

Platelet Counts and TPO

The peripheral platelet counts were significantly lower in patients during the acute phase compared with controls ($P < .0001$; Table 1) and were significantly lower in DHF patients compared with DF patients ($P < .05$; Table 1). The low baseline platelet counts during the acute phase increased significantly to normal levels during the early convalescence and convalescence phases ($P < .0001$; Figure 1*A*). In contrast, serum TPO levels were significantly higher in patients during the acute phase compared with controls ($P < .0001$; Table 1). Serum TPO levels decreased significantly during the early convalescence

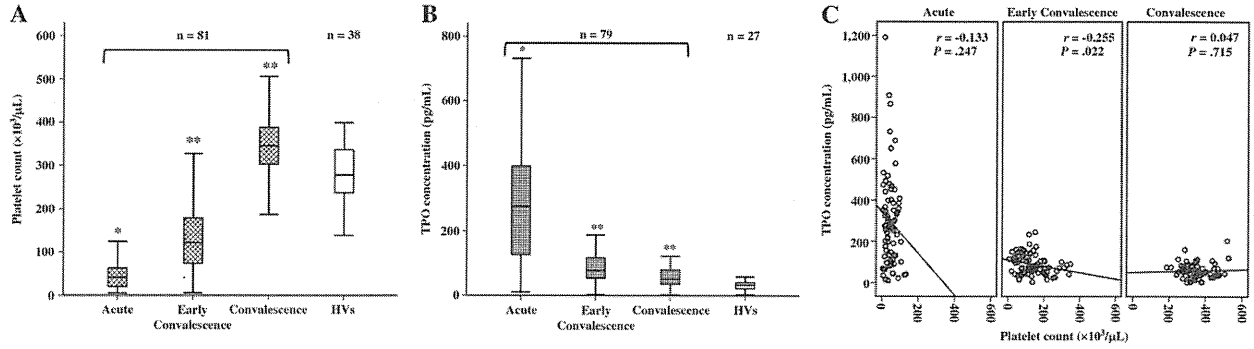

Figure 1. Kinetics of platelet counts (*A*) and serum thrombopoietin (TPO) levels (*B*) in patients during the acute, early convalescence, and convalescence phases and in healthy volunteers (HVs), and the relationship between platelet counts and serum TPO levels (*C*) in patients during the acute, early convalescence, and convalescence phases. $*P < .0001$ (vs HVs). $**P < .0001$ (vs acute).

and convalescence phases ($P < .0001$; Figure 1*B*). No correlation was found between platelet counts and serum TPO levels during the acute and convalescence phases, but a weak inverse correlation was found between these 2 parameters during the early convalescence phase (Figure 1*C*).

Platelet Apoptosis

Representative density plots of the frequency of platelet apoptosis, as measured using annexin V binding and levels of active caspase 3, are shown for controls and patients with DF or DHF during the acute phase (Figure 2). Platelet apoptosis determined via annexin V binding and the levels of active caspase 3 was significantly increased in patients during the acute phase compared with controls ($P < .0001$; Table 1). Platelet apoptosis was also significantly greater in DHF patients than in DF patients ($P < .05$; Table 1). The increased apoptosis of the platelets isolated from patients, as evidenced by both annexin V binding and the levels of active caspase 3, decreased significantly during the early convalescence and convalescence phases ($P < .01$ for early convalescence phase, $P < .0001$ for convalescence phase; Figure 3*A* and 3*B*). The number of apoptotic platelets exposing PS was significantly elevated in patients during the early convalescence ($P < .0001$) and convalescence phases ($P < .0001$) but not during the acute phase ($P = .173$) compared with that observed in controls (Figure 3*C*).

Platelet Phagocytosis and Correlation With Platelet Apoptosis

Platelet phagocytosis was significantly increased in patients compared with controls ($P < .0001$; Table 1) and differed significantly between patients with DF and those with DHF ($P < .05$; Table 1). The significantly elevated platelet phagocytosis values during the acute phase returned to normal levels during the early convalescence ($P < .01$) and convalescence phases ($P < .0001$; Figure 4*A*). During the acute phase, significant correlations were observed between platelet phagocytosis and platelet apoptosis ($P < .0001$ for both annexin V binding and active caspase 3; Figure 4*B* and 4*C*). Weak but significant correlations between these 2 parameters were also found during the early convalescence phase; however, during the convalescence phase, they were only significant for active caspase 3.

Inhibition of Platelet Phagocytosis

Next, we determined whether the apoptotic platelets were phagocytosed by macrophages via the PS-recognition pathway

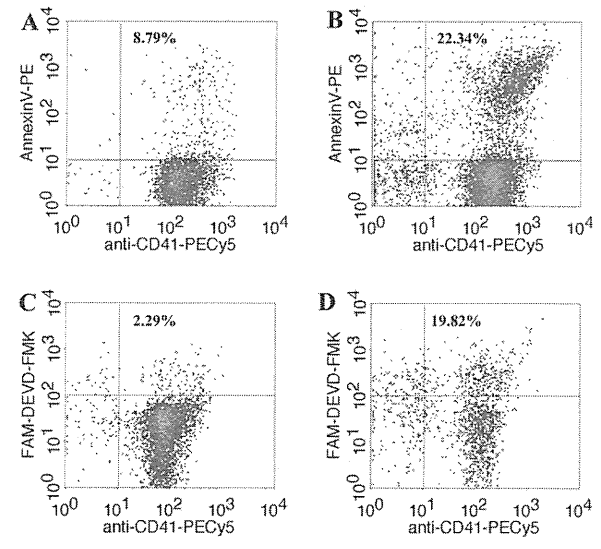

Figure 2. Representative density plots of the frequency of apoptosis measured using phycoerythrin (PE)–conjugated annexin V binding in platelets from a healthy volunteer (*A*) and from a patient with dengue hemorrhagic fever (*B*) and measured using levels of active caspase 3 in platelets from a healthy volunteer (*C*) and from a patient with dengue fever (*D*). The upper-right quadrants show cells positive for anti-CD41 + annexin V or anti-CD41 + FAM-DEVD-FMK, which were considered to be apoptotic.

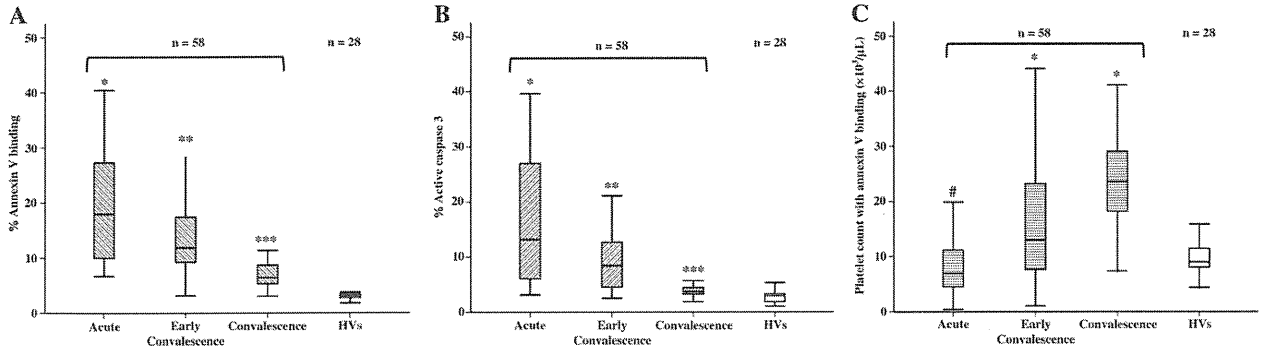

Figure 3. Kinetics of percentage annexin V binding (*A*), active caspase 3 (*B*), and platelet counts with annexin V binding (*C*) in 58 patients during the acute, early convalescence, and convalescence phases and in 28 healthy volunteers (HVs). Platelet counts with annexin V binding were calculated based on the platelet counts and the percentage of apoptotic platelets was determined using annexin V binding. $^{*}P < .0001$ (vs HVs). $^{**}P < .01$ (vs acute). $^{***}P < .0001$ (vs acute). $^{\#}P = .173$ (vs HVs).

by pretreating platelets with the D89E mutant protein to block the phagocytosis of apoptotic platelets. This treatment led to a significant inhibition of platelet phagocytosis in 7 patients with DHF (Figure 5*A*) and 17 patients with DF (Figure 5*B*) during the acute and early convalescence phases. In contrast, no inhibition of phagocytosis was observed in patients during the convalescence phase or in controls (Figure 5*C*).

In Vitro Platelet Apoptosis and Phagocytosis of Platelets

The apoptosis of platelets pretreated with CTR increased significantly in medium containing DV4 after incubation for 72 hours and 96 hours ($P < .0001$) compared with that of platelets pretreated with CTR observed in medium only or in medium containing JEV (Figure 6*A*). Pretreatment with a caspase 3 inhibitor reduced significantly the apoptosis of platelets cultured in medium containing DV4 or JEV or in medium only after incubation for 72 hours and 96 hours ($P < .05$) compared with platelets pretreated with CTR. Our Western blot results confirmed the enhanced activation of caspase 3 in platelets cultured in medium containing DV4 compared with platelets cultured in medium only or in freshly isolated platelets (Figure 6*B*). The phagocytosis of platelets pretreated with CTR cultured in medium containing DV4 for 72 hours was significantly higher than that observed in platelets pretreated with CTR cultured in medium only ($P < .0001$; Figure 6*C*). Pretreatment with a caspase 3 inhibitor reduced significantly the phagocytosis of platelets pretreated with CTR cultured in medium only ($P < .01$) or in medium containing DV4 ($P < .0001$). Culture in the presence of DV1, DV2, or DV3 increased the apoptosis of platelets pretreated with CTR similarly after incubation for 72 hours and 96 hours compared with that observed in medium only ($P < .0001$; Figure 6*D*). Pretreatment with a caspase 3 inhibitor reduced significantly the apoptosis of platelets cultured in medium containing DV1,

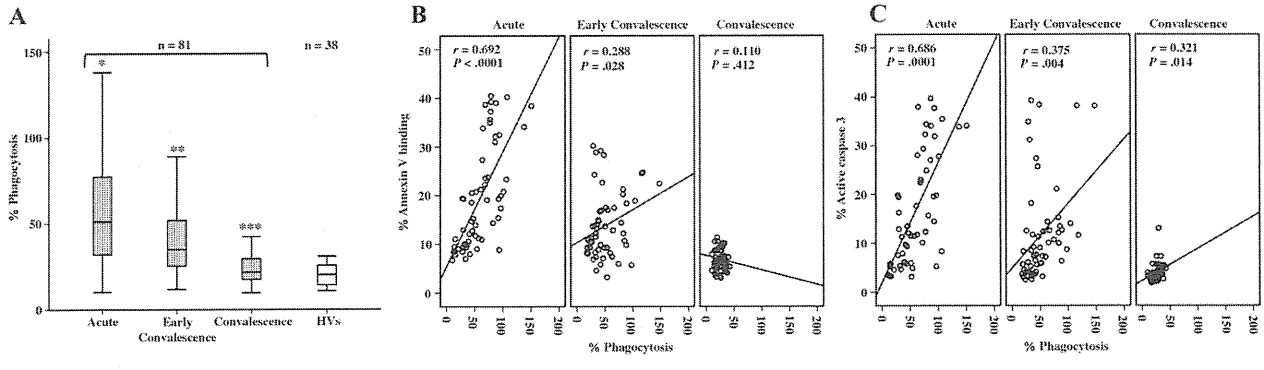

Figure 4. Kinetics of percentage platelet phagocytosis in 81 patients during the acute, early convalescence, and convalescence phases and in 38 healthy volunteers (HVs) (*A*), and relationship between the percentage of platelet phagocytosis and the percentage of annexin V binding (*B*) or active caspase 3 (*C*) during the acute, early convalescence, and convalescence phases in 58 patients. $^{*}P < .0001$ (vs HVs). $^{**}P < .01$ (vs acute). $^{***}P < .0001$ (vs acute).

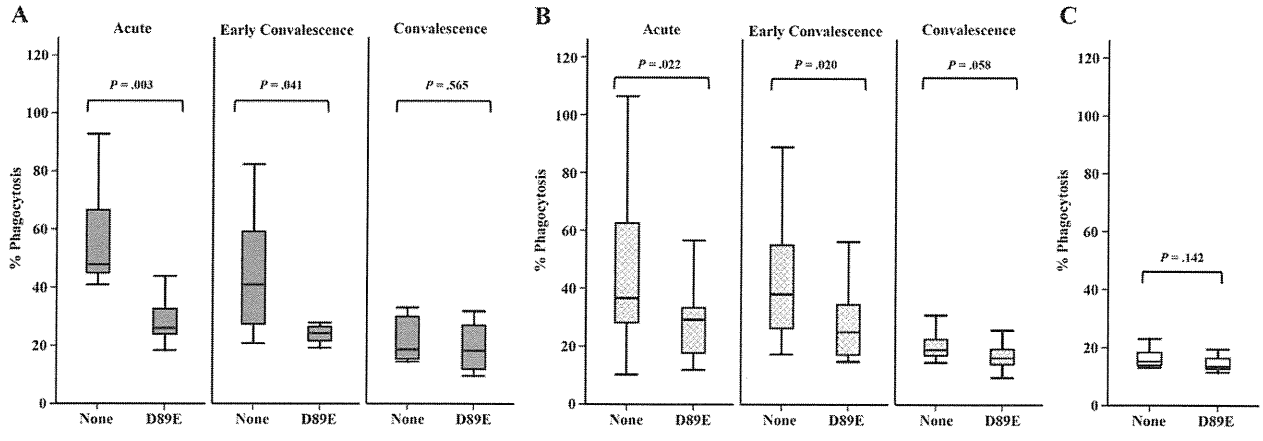

Figure 5. Inhibition of platelet phagocytosis by pretreatment with mutant MFG-E8 (D89E) in platelets from patients with dengue hemorrhagic fever (*A*; n = 7) or dengue fever (*B*; n = 17) and from healthy volunteers (*C*; n = 20). *P* values shown were calculated in relation to absence of treatment.

DV2, or DV3 or in medium only after incubation for 72 hours and 96 hours compared with platelets pretreated with CTR ($P < .0001$).

DISCUSSION

In this study, we demonstrated a significant increase in platelet apoptosis in patients during the acute and early convalescence phases of secondary DV infections compared with the increase observed in patients during the convalescence phase or in controls. Although the frequency of apoptosis has been reported as <0.1% in peripheral blood mononuclear cells of pediatric patients with DF or DHF during the acute phase [21], the frequency of apoptotic platelets was >10% in patients during the acute and early convalescence phases. Platelet apoptosis was significantly higher in DHF patients than in DF patients. Because plasma viremia is higher in DHF patients than in DF patients during the acute phase [22], the induction of platelet apoptosis is presumably attributable to dengue viremia. Although a recent study reported that erythrocyte-derived microvesicles released PS to the surface of nucleated cells during erythrocyte lysis [23], we did not use a procedure of erythrocyte lysis to separate platelets in this study. Therefore, the results in our study may not involve this false marking with PS as apoptotic.

Our in vitro assays demonstrated that all serotypes of DV enhanced the spontaneous apoptosis of human platelets. The phagocytosis of cultured platelets after exposure to DV4 was also increased. In contrast, no enhancement of spontaneous apoptosis was found in JEV-exposed platelets. This finding suggests the DV-specific induction of platelet apoptosis and a direct interaction between DV and platelets, although the precise nature of these molecular interactions remains unclear. Detection of dengue virus RNA and the electron microscopy–based finding of DV-like particles in platelets isolated from dengue patients may, in part, support our in vitro results [17, 24]. Previous studies have reported increased levels of tumor necrosis factor α in the sera of DHF patients [25, 26] and suggested that this enhanced apoptosis of platelets is triggered by the extrinsic death pathway in the blood circulation of these patients [16]. Because the in vitro exposure of human platelets to DV2 led them to bind positively in the presence of a virus-specific antibody [27], DV may bind to platelets in patients with secondary DV infections who have increased serum IgG levels that recognize the infecting DV serotype.

Some similar biochemical pathways are used during platelet activation and platelet apoptosis [28]. In activated platelets, P-selectin is translocated from the α-granule and exposed on the platelet surface [29]. A previous in vitro study reported that exposure of DV2 increased expression of P-selectin, which is a marker of platelet activation and fibrinogen-binding properties in platelets [30]. In our preliminary study performed in 2008, we assessed the extent of the expression of P-selectin in 19 patients with secondary DV infections and 15 controls. The frequency of P-selectin expression on platelets was increased significantly in patients during the acute phase compared with controls, increased further during the early convalescence phase, and decreased slightly during the convalescence phase (data not shown). Therefore, the kinetics of the expression of P-selectin were distinct from those of platelet apoptosis observed in the present study, which is in agreement with a previous report of an in vitro study [31].

Platelet apoptosis correlated significantly with the platelet phagocytosis in our patients, as assessed using an ex vivo assay. This increased platelet phagocytosis was inhibited significantly in patients during the acute and early convalescence phases by pretreatment with the D89E mutant protein. These findings suggest that apoptotic platelets are phagocytosed via a PS-recognizing pathway in the systemic circulation of these

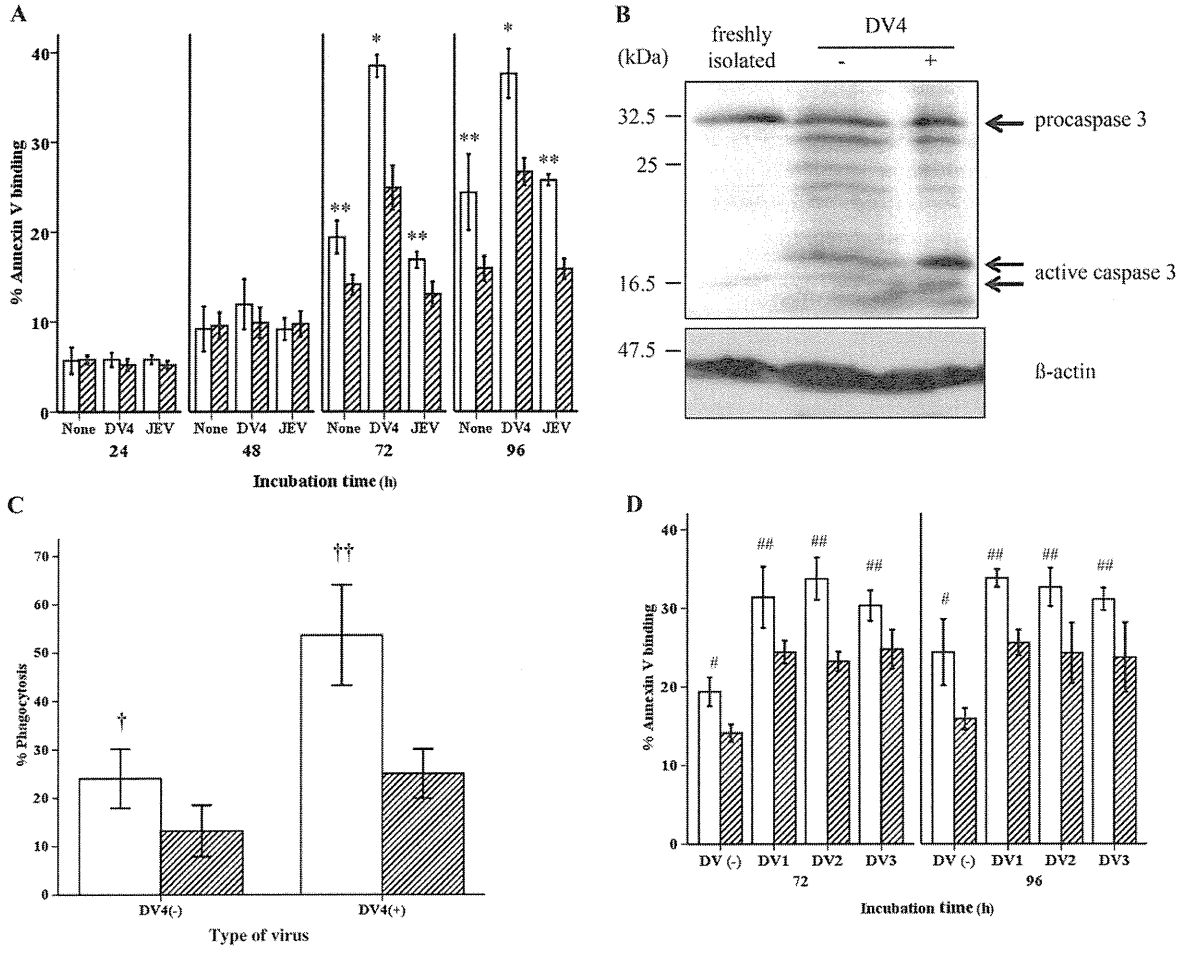

Figure 6. Effect of dengue virus 4 (DV4) or Japanese encephalitis virus (JEV) on platelet apoptosis determined using annexin V binding (*A*) and activation of caspase 3 (*B*) and on platelet phagocytosis (*C*) in vitro, and effect of DV1, DV2, or DV3 on platelet apoptosis in vitro (*D*). Percentage of apoptosis of platelets pretreated with a caspase inhibitor negative control (*open bar*) or platelets pretreated with a caspase 3 inhibitor (*filled bar*) from healthy donors cultured in L-15 medium containing DV of each serotype or JEV or in medium only. Percentage of phagocytosis of platelets pretreated with a caspase inhibitor negative control (*open bar*) or platelets pretreated with a caspase 3 inhibitor (*filled bar*) cultured in L-15 medium containing DV4 or in medium only for 72 h. $^{*}P < .0001$ (vs pretreatment with a caspase inhibitor negative control in medium only or in medium containing JEV or pretreatment with a caspase 3 inhibitor in medium containing DV4). $^{**}P < .05$ (vs pretreatment with a caspase 3 inhibitor in medium only or in medium containing JEV). $^{\dagger}P < .01$ (vs pretreatment with a caspase 3 inhibitor in medium only). $^{\dagger\dagger}P < .0001$ (vs pretreatment with a caspase inhibitor negative control in medium only or pretreatment with a caspase 3 inhibitor in medium containing DV4). $^{\#\#}P < .0001$ (vs pretreatment with a caspase inhibitor negative control in medium only or pretreatment with a caspase 3 inhibitor in medium containing DV after 72 h and 96 h incubation). Data are expressed as the mean (SD) (9 wells per group from 3 independent experiments).

patients. A recent report of the engulfment of platelets by monocytes in a nonhuman primate model of DV infection supports the present findings in our patients [32].

It is important to determine the role of the accelerated phagocytosis of apoptotic platelets in thrombocytopenia in this disease. With an adult blood volume of 5.0 L, approximately 5.0×10^{10} apoptotic platelets may be cleared from the blood circulation each day because the concentration of apoptotic platelets exposing PS was approximately 1.0×10^{10}/L of blood in controls (Figure 3*C*). In contrast, the concentration of apoptotic platelets in our patients was 0.7 (0.4–1.10) $\times 10^{10}$/L of blood in the acute phase, 1.3 (0.8–2.3) $\times 10^{10}$/L of blood in the early convalescence phase, and 2.4 (0.8) $\times 10^{10}$/L of blood in the convalescence phase. Collectively, our data indicate that approximately 6.5–12.0 $\times 10^{10}$ apoptotic platelets, which is 1.3–2.4 times greater than that observed in controls, may be cleared from the blood circulation in patients during the early convalescence and convalescence phases. However, the accelerated clearance of apoptotic platelets by macrophages appeared to be overcome rapidly by enhanced thrombopoiesis

because platelet production can increase >10-fold under the conditions of increased demand [33]. Accelerated platelet clearance, therefore, may not have affected the severity of thrombocytopenia in our patients.

Although the patients enrolled in this study were diagnosed based on the WHO case definition of 1997 [18], Deen et al emphasized the necessity of a reassessment of this WHO case definition because of several difficulties regarding its practical use [34]. Based on this background, the new WHO guidelines, published in 2009, include cases with severe bleeding and severe organ involvement [35], and a recent revised and expanded edition of dengue guidelines, published in 2011, includes dengue fever without unusual hemorrhage and expanded dengue syndrome [36]. However, our cohort did not include such cases with unusual manifestations; thus, the application of these new guidelines does not alter the findings of the present study. Further studies are required to determine the role of apoptotic platelet clearance in patients with primary infection or dengue shock syndrome and unusual manifestations classified in the new guidelines [35, 36].

TPO is produced at a constant rate by the liver and enters the circulation where TPO receptors on platelets bind to and degrade this protein. Therefore, serum TPO levels correlate inversely with platelet production rate [37]. The levels of TPO were increased in the sera of our patients during the acute phase, which is consistent with the results of a previous study [38]. This finding may be explained by the suppression of megakaryocytopoiesis and reduction in platelet production, which have been reported in DHF patients in earlier studies [3, 4]. However, we found no correlation between platelet counts and serum TPO levels during the acute and convalescence phases in this study. The regulation of serum TPO levels in these patients appears to be complex and its underlying mechanisms remain uncertain.

In conclusion, the present data suggest the increased clearance of DV-induced apoptotic platelets by macrophages primarily via a PS-recognizing pathway in patients with secondary DV infection. To the best of our knowledge, this is the first evidence of platelet apoptosis and accelerated clearance of apoptotic platelets in this disease.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Population-Based Study of *Streptococcus suis* Infection in Humans in Phayao Province in Northern Thailand

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Abstract

Background: *Streptococcus suis* infection in humans has received increasing worldwide recognition.

Methods and Findings: A prospective study of *S. suis* infection in humans was conducted in Phayao Province in northern Thailand to determine the incidence and the risk behaviors of the disease in this region in 2010. Thirty-one cases were confirmed. The case fatality rate was 16.1%, and the estimated incidence rate was 6.2 per 100,000 in the general population. The peak incidence occurred in May. The median age of the patients was 53 years and 64.5% were men. Consumption of raw pork products was confirmed in 22 cases and the median incubation period (range) was 2 days (0–11) after consumption of raw pork products. Isolates from 31 patients were confirmed as serotype 2 in 23 patients (74.2%) and serotype 14 in eight patients (25.8%). The major sequence types (STs) were ST1 (n = 20) for serotype 2 and ST105 (n = 8) for serotype 14. The epidemiological analysis suggested three possible clusters, which included 17 cases. In the largest possible cluster of 10 cases in Chiang Kham and its neighboring districts in May, the source of infection in four cases was identified as a raw pork dish served at the same restaurant in this district. Microbiological analysis confirmed that three of four cases associated with consumption of raw pork at this restaurant were attributable to an identical strain of serotype 2 with ST1 and pulsotype A2.

Conclusions: Our data suggest a high incidence rate of *S. suis* infection in the general population in Phayao Province in 2010 and confirm a cluster of three cases in 31 human cases. Food safety control should be strengthened especially for raw pork products in northern Thailand.

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Introduction

Streptococcus suis is a zoonotic pathogen that can cause invasive infection in humans who have close contact with infected pigs or contaminated pork-derived products. The numbers of reported human cases, especially in Southeast Asian countries, have increased dramatically in the past few years [1–3]. Although serotype 2 is the most prevalent in humans, human cases involving serotypes 1, 4, 14 and 16 have been reported [1–5]. In a retrospective study in 2006–2008 in Thailand, *S. suis* infection was confirmed in bacterial cultures of blood or cerebrospinal fluid (CSF) from 179 patients. These isolates were determined to be serotype 2 for 165 cases (92.2%), serotype 14 for 12 cases (6.7%), and one case each (0.6%) of serotypes 5 and 24 [4–6]. Human infection with serotype 2 was sporadic, with a case fatality rate of 9.5% in adults, and most of these cases were located primarily in northern Thailand [4].

The population of the 17 provinces in northern Thailand was 11,788,684 in 2010 (Figure 1A) [7]. Some local residents have a traditional custom of consuming raw pork dishes such as "Loo" (raw pork meat and blood), "Lap" (raw pork meat), and fermented raw pork in this region. An outbreak of *S. suis* infection including 29 laboratory-confirmed cases occurred in the Phu Sang district, Phayao Province, in northern Thailand in April and May of 2007 (Figure 1B) [8] A major route of transmission during this outbreak was the consumption of raw pig blood. This province is located

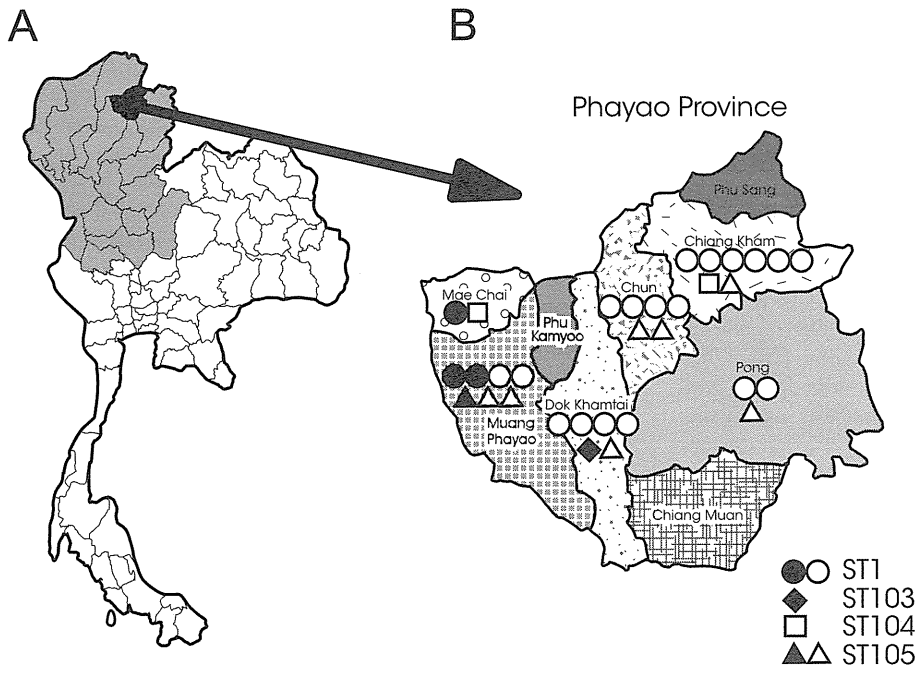

Figure 1. Location of the study site and distribution of human isolates. (**A**) Location of Phayao Province in northern Thailand. (**B**) Distribution and sequence typing of 31 human isolates of *Streptococcus suis* in Phayao Province in 2010 (B). One symbol is one case. Closed symbols denote fatal cases, and open symbols denote nonfatal cases.
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close to the border with the Lao People's Democratic Republic, and the population of this province was 486,304 in 2010 [7]. Although previous studies reported that human cases of *S. suis* infection are associated with the recent consumption of raw pork products in northern Thailand and Vietnam [2–6,8–11], the annual incidence rate of this disease in this region remains unknown.

In this study, we conducted a population-based study of *S. suis* infection in humans to determine the incidence rate of this disease in Phayao Province in 2010. We also investigated the risk behaviors of this disease and the possible clustering of cases in relation to the risk behaviors.

Methods

Human cases

We organized a network for the surveillance of *S. suis* infection in humans that includes the Phayao Public Health Office and two tertiary hospitals (Phayao Provincial Hospital and Chiang Kham General Hospital), and five district hospitals (Mae Chai Hospital, Chiang Muan Hospital, Dok Khamtai Hospital, Chun Hospital, and Pong Hospital); the districts within this province are shown in Figure 1B. We enrolled hospitalized patients with sepsis or bacterial meningitis when a biochemical test suggested the presence of *S. suis* in isolates from blood or CSF, and prospectively investigated the clinical and epidemiological features of the enrolled cases at these seven hospitals between January to December of 2010.

The clinical information of the enrolled case was recorded by attending physicians at a hospital in a network for the surveillance of *S. suis* infection in Phayao Province. The clinical information included the date of onset of illness and the hospital admission, and the risk behaviors, such as occupational exposures, the recent contact with pigs or raw pork products and the recent consumption of raw pork products. For the cases with the recent contact with pigs or raw pork products, the date and the location of exposure were recorded. For the cases with the recent consumption of raw pork products, the date and place of consumption of raw pork products and the type of dishes containing raw pork products were recorded. The clinical categories included meningitis and nonmeningitis based on the definition previously described [4]. The meningitis category involved confirmed meningitis, bacteremic meningitis, and probable meningitis. All patients in the meningitis category had typical meningeal signs, such as neck stiffness and an acute onset. Bacteremic meningitis was defined as a positive result in both the CSF and blood cultures, confirmed meningitis was defined as a positive culture in the CSF only, and probable meningitis was defined as a positive blood culture. The nonmeningitis category included the clinical manifestations of sepsis and sepsis with focal signs other than meningitis (septic arthritis or bacteremic pneumonia). Sepsis was defined as systemic inflammatory response syndrome with a positive blood culture.

The possible clustered cases were defined as human cases of laboratory-confirmed *S. suis* infection in combination with the recent close contact with pigs or raw pork products or with the recent consumption of raw pork products in the same or neighboring districts within 14 days of each onset of illness. This incubation period was based on a previous report of a human *S. suis* outbreak in Sichuan, China, which showed that the interval between exposure and onset is 1–14 days [12]. This population-based study of *S. suis* infection in humans was reviewed and approved by the Ethics Committees of the Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. This study was conducted according to the principles expressed in the Declaration of Helsinki. The patient or guardian provided written informed consent for all cases. This study was registered at the UMIN Clinical Trial Registry (UMIN000006449).

Microbiological study

The isolates were subjected to the following biochemical tests: API Strep (bioMérieux, Durham, NC, USA) and *S. suis*-specific and *S. suis* serotype 2- or 1/2–specific polymerase chain reaction [4,13]. The final serotype of all strains was confirmed by coagglutination tests using rabbit antisera (Statens Serum Institute, Copenhagen, Denmark).

Multilocus sequence typing (MLST) was performed as described by King et al. [14], with a modification for *mutS* as described by Rehm et al. [15]. MLST alleles and the resulting sequence type (ST) were assigned using the *S. suis* MLST database, which can be accessed at http://ssuis.mlst.net. Pulsed-field gel electrophoresis (PFGE) was performed as described previously [16]. The pulsotypes were designated as previously described [4], and assigned to clusters of isolates with >80% similarity within the dendrogram.

Statistical analysis

The clinical characteristics including male sex, age, risk factor, the days from the consumption of raw pork products to the onset of illness, the days from the onset of illness to the admission between fatal and nonfatal cases were compared using Fisher's exact test or Mann–Whitney *U* test with SPSS version 15.0 software. Data were considered significant for p values<0.05.

Results

Patients

The locations of nine districts in Phayao Province and the distribution of the 31 cases in these districts are shown in Figure 1B. No case was found in the districts of Phu Sang, Phu Kamyoo, and Chiang Muan. Fatal cases were found in the districts of Muang Phayao, Dok Khantai, and Mae Chai. There was no relationship between geographical distribution of cases and the location of fatal cases. The monthly incidence of the 31 cases in each district is shown in Figure 2. The peak incidence occurred in May, and 23 cases (71.9%) were detected between April and June.

The clinical features of the 31 patients admitted with *S. suis* infection in Phayao Province between January and December 2010 are shown in Table 1. The median age (range) of these patients was 53 years (26–74) of which 64.5% were men and 35.5% were women. Five of the 31 cases (16.1%) were fatal. Recent exposure to pigs or raw pork products was noted in two cases (6.5%). One case occurred in a pork meat seller (Case 21 in Table 2) and was associated with the daily occupational exposure to the raw pork products at the wet market. Another case (Case 27 in Table 2) had the daily exposure to pigs bred at home.

Because 30 cases were confirmed in the general population of Phayao Province in 2010, the incidence rate of this disease in humans was 6.2 per 100,000 (30 in 486,304) of the general population in this province in 2010. Before the onset of illness, a recent history of consumption of raw pork products was confirmed for 22 (71.0%) of the 31 cases. A pig breeder (Case 27) also had a recent history of raw pork consumption. No information was available about the recent consumption of raw pork products or exposure to pigs or raw pork products in the remaining eight cases.

The clinical manifestations in the 31 patients included fever (n = 27; 87.1%), headache (n = 19; 61.3%), hearing loss (n = 12; 38.7%), altered consciousness (n = 9; 29.0%), and diarrhea (n = 6; 19.4%). The comorbid illnesses of these patients included alcoholic liver cirrhosis (n = 4; 12.9%), hypertension (n = 3, 9.7%), diabetes mellitus (n = 1; 3.2%), rheumatoid arthritis (n = 1; 3.2%), aplastic anemia (n = 1; 3.2%), and spinal canal stenosis (n = 1; 3.2%). No comorbid illness was found in 20 patients (64.5%). None of the demographic features, including the risk behavior of recent consumption of raw pork products and recent exposure to pigs or raw pork products, was significantly associated with a fatal outcome (Table 1). The median interval (range) between the consumption and onset of illness was 2 days (0–11) for 22 patients. The median period (range) from the onset of illness to admission was 2 days (0–14) for 31 patients. The interval from the onset of illness to admission was not associated significantly with a fatal outcome, although the interval tended to be longer in the fatal cases than in the nonfatal cases. The meningitis category (n = 20; 64.5%) included five cases of confirmed meningitis, nine cases of

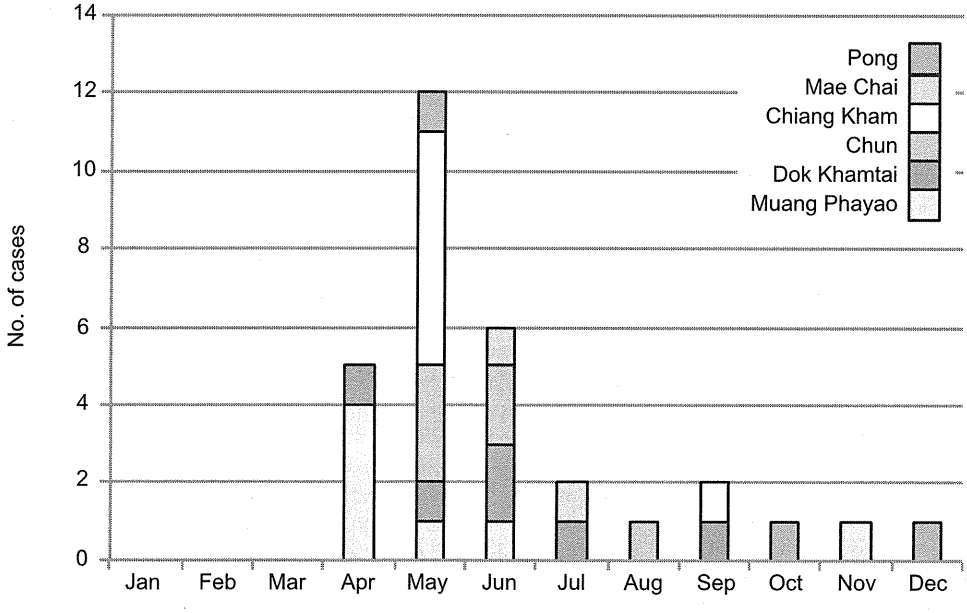

Figure 2. Monthly distribution of human cases of *Streptococcus suis* infection in each district in Phayao Province in 2010.
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Table 1. Clinical characteristics of 31 human cases of *Streptococcus suis* infection in Phayao Province, 2010.

Characteristics	All reported cases	Nonfatal case, n = 26; 83.9%	Fatal case, n = 5; 16.1%	*p*-value
Demographic				
Male, no. of cases (%), n = 31	20 (64.5)	18 (69.2)	2 (40)	0.317
Age, median (range), n = 31	53 (26–74)	52 (26–74)	64 (36–72)	0.115
Risk behavior, no. of cases (%)				
Recent consumption of raw pork products, n = 31	22 (71.0)	20 (76.9)	2 (40)	0.131
Recent contact with pigs or raw pork products, n = 31	2 (6.5)	2 (7.7)	0 (0)	1
Days from the consumption of raw pork products to the onset of illness median (range), n = 22	2 (0–11)	2 (0–11)*	1.5 (1–2)**	0.623
Days from the onset of illness to the admission median (range), n = 31	2 (0–14)	2 (0–7)	4 (0–14)	0.176

*n = 20,
**n = 2.
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bacteremic meningitis, and six cases of probable meningitis. The nonmeningitis category (n = 11; 35.5%) included five cases of septic arthritis and six cases of sepsis.

Clustered cases

We next examined whether the clustered cases that were linked epidemiologically and caused by an identical strain, were included in the 31 cases. The clinical, epidemiological and microbiological features of 31 human cases of *S. suis* infection is shown in Table 2. In 22 patients with a recent history of consumption of raw pork products, these products were consumed at home by 14 patients and at 5 different restaurants by eight patients. The most frequent dish (14/22 cases; 63.6%) was "Loo". Three possible clusters including 17 cases associated with recent consumption of raw pork products or the recent exposure to pigs or raw pork products were found based on the case definition in the 31 cases. A possible cluster in the Muang Phayao district found in April included three cases (shown as PC I). Another possible large cluster including 10 cases was found in the districts of Chiang Kham, Chun, and Pong in May (shown as PC II). The other possible cluster including four cases was found in the districts of Dok Khamtai and Chun between May and June (shown as PC III). Interestingly, four patients visited restaurant C and consumed "Loo" in Chiang Kham district between May 8 and 15, 2010. These four patients had febrile illness 1–4 days after consuming "Loo" at this restaurant. By contrast, no epidemiological linkage was found in the other 13 cases in three possible clusters.

Isolates of *S. suis*

S. suis was isolated from all 31 patients. Of the 31 isolates, 23 (74.2%) were serotype 2 and the other eight (25.8%) were serotype 14 (Table 2). The sequence typing of serotype 2 isolates were ST1 for 20 isolates (64.5%), ST104 for two isolates (6.5%), and ST103 for one isolate (3.2%). All eight serotype 14 isolates were ST105 (25.8%). In four patients in the possible large cluster (PC II in Table 2) in Chiang Kham and its neighboring district with a history of visiting restaurant C, serotype 2 strain with ST1 and pulsotype A2 was isolated from three cases, and serotype 2 strain with ST1 and pulsotype A was isolated from one case.

Discussion

In this study, we confirmed 31 human cases of *S. suis* infection with a case fatality rate of 16.1% in Phayao Province in 2010. This case fatality rate is equivalent to that recorded previously in Thailand [4,11,12]. To exclude the possibility that human cases of *S. suis* infection in residents of Phayao Province were detected in hospitals in the surrounding three provinces of Chiang Rai, Lampang, and Nan, we investigated all human cases in these provinces through the hospital network surveillance system for *S. suis* infection organized by the Thai NIH in 2010 [17]. Because no human cases from Phayao Province were found in these three provinces in this surveillance, our data represent a population-based study of *S. suis* infection in humans in this province.

The incidence rate (6.2 per 100,000) in the general population in Phayao Province in 2010 is 69 times higher than that (0.09 per 100,000) in Hong Kong [18], which is the sole available data for the general population in Southeast Asian countries. By contrast, the incidence rate of this disease is as low as 0.002/100,000 in the general population in a developed country such as The Netherlands [19]. Our present data suggest that the highest incidence rate of this disease among adults in the general population in this region is associated with the habitual behavior of consuming raw pork products. Given the incidence rate of this disease and the population in northern Thailand, the estimated number of human cases can be calculated as 730 per year in this region.

The disease incidence peaked during the rainy season (June to August) in a retrospective study between 2006 and 2008 in all 76 provinces of Thailand [4]. By contrast, the peak incidence was May 2010 in our current study. A previous outbreak in the Phu Sang district, Phayao Province, was also found during April and May in 2007 [8]. The shift of the peak incidence to April and May might be related to the Songkran Festival (a traditional new year festival in Thailand) in April and other harvesting festivals during this period in this region.

A recent case–control study in southern Vietnam reported that eating undercooked pig blood or intestine within 2 weeks of the appearance of infection was the most important risk factor [20]. In our study, we also confirmed that more than 70% of cases with *S. suis* infections were associated with the recent consumption of raw pork products. Importantly, the estimated incubation period for this disease after oral consumption of raw pork products was only 2 days. This finding strongly suggests that the oral consumption of raw pork products is the major transmission route. A previous study of an *S. suis* outbreak in Sichuan, China, similarly reported a median interval of 2.2 days between exposure and onset of infection, although the transmission route in this outbreak was direct contact with the blood or tissues of sick or dead pigs [12].

Table 2. Clinical, epidemiological and microbiological features of 31 human cases of *Streptococcus suis* infection in Phayao Province, 2010.

No.	Age	Sex	District	Contact with pigs or raw pork products	Consumption of raw pork products				Day of onset	Outcome	Serotype	Pulsotype*	MLST		Possible cluster
					Consumption	Date	Place	Type of product					ST complex	ST	
1	41	M	Muang Phayao	No	Yes	2 Apr	Home	Loo	5 Apr	Alive	2	A3	1	1	PC I
2	46	M	Muang Phayao	No	Yes	15 Apr	Restaurant A	Loo	17 Apr	Alive	2	A	1	1	PC I
3	72	M	Muang Phayao	No	Yes	20 Apr	Restaurant B	Loo	22 Apr	Dead	2	A	1	1	PC I
4	63	F	Muang Phayao	No	No	-			28 Apr	Dead	14	J	1	105	
5	51	M	Muang Phayao	No	No	-			15 May	Alive	14	J	1	105	
6	52	M	Dok Khamtai	No	No	-			24 Apr	Alive	14	J	1	105	
7	50	M	Chiang Kham	No	Yes	30 Apr	Home	Boiled intestine	1 May	Alive	14	J	1	105	PC II
8	56	M	Chiang Kham	No	Yes	8 May	Restaurant C	Loo	10 May	Alive	2	A	1	1	PC II
9	65	M	Chiang Kham	No	Yes	12 May	Restaurant C	Loo	13 May	Alive	2	A2**	1	1	PC II
10	49	M	Chiang Kham	No	Yes	12 May	Restaurant C	Loo	16 May	Alive	2	A2**	1	1	PC II
11	26	F	Chiang Kham	No	Yes	15 May	Restaurant C	Loo	17 May	Alive	2	A2**	1	1	PC II
12	53	M	Chiang Kham	No	Yes	12 May	Home	Lap	14 May	Alive	2	A2	1	1	PC II
13	59	M	Chun	No	Yes	6 May	Home	Lap	7 May	Alive	2	A2	1	1	PC II
14	57	M	Chun	No	Yes	12 May	Home	Fermented raw pork	14 May	Alive	14	J	1	105	PC II
15	54	M	Chun	No	Yes	10 May	Home	Fermented raw pork	14 May	Alive	14	J	1	105	PC II
16	45	F	Pong	No	Yes	3 May	Home	Fermented raw pork	10 May	Alive	14	J	1	105	PC II
17	72	F	Dok Khamtai	No	No	-			10 Jun	Alive	2	A1	1	1	
18	42	F	Dok Khamtai	No	Yes	26 May	Home	Loo	28 May	Alive	2	A1	1	1	PC III
19	41	M	Dok Khamtai	No	Yes	1 Jun	Restaurant D	Loo	3 Jun	Alive	2	A1	1	1	PC III
20	50	M	Chun	No	Yes	1 Jun	Home	Loo	12 Jun	Alive	2	A2	1	1	PC III
21	60	F	Chun	Yes	No	-			19 Jun	Alive	2	A2	1	1	PC III
22	52	F	Muang Phayao	No	No	-			15 Jun	Alive	14	J	1	105	
23	74	F	Mae Chai	No	No	-			20 Jun	Alive	2	H	225	104	
24	69	F	Mae Chai	No	No				20 Jul	Dead	2	A	1	1	
25	66	M	Dok Khamtai	No	Yes	2 Jul	Home	Loo	4 Jul	Alive	2	A5	1	1	
26	55	M	Chun	No	Yes	30 Jul	Home	Lap	1 Aug	Alive	2	A2	1	1	
27	34	M	Chiang Kham	Yes	Yes	30 Aug	Home	Loo	1 Sep	Alive	2	H	225	104	

Table 2. Cont.

No.	Age	Sex	District	Contact with pigs or raw pork products	Consumption of raw pork products				Day of onset	Outcome	Serotype	Pulsotype*	MLST		Possible cluster
					Consumption	Date	Place	Type of product					ST complex	ST	
28	36	M	Dok Khamtai	No	Yes	11 Sep	Home	Loo	12 Sep	Dead	2	K	29	103	
29	47	F	Pong	No	Yes	20 Oct	Restaurant E	Loo	21 Oct	Alive	2	A	1	1	
30	64	F	Muang Phayao	No	No	-			23 Nov	Dead	2	A1	1	1	
31	59	M	Pong	No	Yes	5 Dec	Home	Lap	6 Dec	Alive	2	A2	1	1	

Three possible clusters in the district of Muang Phayao (PC I), the districts of Chiang Kham, Chun and Pong (PC II), and the districts of Dok Khantai and Chun (PC III) are shown.

*The pulsotype was designated as previously described [4]. A2**, Serotype 2 with pulsotype A2 was the causative pathogen of a cluster of three cases. MLST, multilocus sequence type; ST, sequence typing.

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No information was available about the recent history of the consumption of raw pork products or exposure to pigs or raw pork products in eight cases (25.8%), which included 6 housewives who might have been exposed unintentionally to the contaminated pork products during cooking.

Of the 31 cases in our study, 23 (74.2%) were caused by serotype 2 and eight (25.8%) were caused by serotype 14. In the previous retrospective study between 2006 and 2008, serotype 14 was confirmed in 12 of 179 human cases (6.7%) of *S. suis* infection in Thailand [5,6]. Of these 12 cases, only one case with serotype 14 was found in Phayao Province in 2007. Our present data suggest that the prevalence of serotype 14 as a cause of human disease has increased in this province since 2009.

Molecular analyses using MLST and PFGE provided evidences of an outbreak of *S. suis* serotype 2 in Sichuan, China [21] and the clonal disseminations of *S. suis* serotypes 2 and 14 among sporadic human cases [4,5]. In the present study, we used MLST and PFGE to investigate whether the 23 human cases found in April to June 2010 contained the clustered cases that were linked epidemiologically through the consumption of raw pork products and caused by an identical pathogen. An obvious spatial and temporal clustering of four cases was found in Chiang Kham district. All four patients (Cases 8–11) consumed "Loo" at restaurant C within the same week in May 2010. The bacteriological analysis confirmed that the four cases were caused by the isolates of serotype 2 with ST1. Of these four isolates, the pulsotypes were A2 for three cases and A for one case. Because the strain with pulsotype A was found to have <80% similarity to strain with pulsotype A2 in the dendrogram [4], this strain with pulsotype A was interpreted to be distinct from pulsotype A2. Our data suggest that the "Loo" at restaurant C were contaminated with two distinct strains of serotype 2 with ST1 and pulsotype A for Case 8 and of serotype 2 with ST1 and pulsotype A2 for Cases 9–11. A cluster comprising three cases caused by an identical serotype 2 strain with ST1 and pulsotype A2 (shown as A2** in Table 2) was confirmed in Chiang Kham district in May, 2010.

Our present data also suggest that the raw pork products consumed by most of our patients were contaminated with *S. suis*. In Phayao Province, most raw pork products are supplied from the local slaughterhouses to the wet markets. A recent study of *S. suis* serotype 2 infection reported that slaughterhouse pigs were the source of infection by *S. suis* serotype 2 in southern Vietnam [22]. A previous study in Hong Kong reported that an increase in bacterial density of *S. suis* in raw pork meats in wet markets occurs in hot and humid climates [23]. Collectively, the poor quality of food safety control for raw pork products at the slaughterhouses and the wet markets in this region are likely to provide the sources of this infection. The surveillance of *S. suis* contamination in pig tonsils collected at slaughterhouses and in the raw pork meats in the wet markets is being investigated in Phayao Province.

Because this study was conducted in hospitals based in Phayao Province, we cannot dismiss the possibility of incomplete coverage of the population. In addition, because the incidence rate was estimated through an observation period of over 1 year, this study should be repeated over a longer period to estimate accurately the incidence rate of this disease.

In conclusion, this population-based study demonstrated a high incidence rate of *S. suis* infection in the general population in Phayao Province, northern Thailand in 2010. Four of 31 human cases were linked epidemiologically to a local restaurant, and three cases were confirmed microbiologically as having been caused by an identical serotype 2 strain with ST1 and pulsotype A2. Combined epidemiological and molecular analyses are helpful for investigating the clustered cases of *S. suis* infection in humans.

Because a large number of human cases of *S. suis* infection is estimated per year in northern Thailand, food safety control of raw pork products should be strengthened in this region.

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Author Contributions

Conceived and designed the experiments: PS SD SH. Performed the experiments: AK SP AY P. Chaiwongsaen TN YA. Analyzed the data: DT AK KO. Contributed reagents/materials/analysis tools: AP P. Loetthong SS PA P. Chiranairadul SL KK P. Luangsuk NW. Wrote the paper: DT KO.

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MAJOR ARTICLE

Identification of Human Papillomavirus Type 58 Lineages and the Distribution Worldwide

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Background. Human papillomavirus type 58 (HPV-58) accounts for a much higher proportion of cervical cancers in East Asia than other types. A classification system of HPV-58, which is essential for molecular epidemiological study, is lacking.

Methods and results. This study analyzed the sequences of 401 isolates collected from 15 countries and cities. The 268 unique concatenated E6-E7-E2-E5-L1-LCR sequences that comprised 57% of the whole HPV-58 genome showed 4 distinct clusters. L1 and LCR produced tree topologies that best resembled the concatenated sequences and thus are the most appropriate surrogate regions for lineage classification. Moreover, short fragments from L1 (nucleotides 6014–6539) and LCR (nucleotides 7257–7429 and 7540–52) were found to contain sequence signatures informative for lineage identification. Lineage A was the most prevalent lineage across all regions. Lineage C was more frequent in Africa than elsewhere, whereas lineage D was more prevalent in Africa than in Asia. Among lineage A variants, sublineage A2 dominated in Africa, the Americas, and Europe, but not in Asia. Sublineage A1, which represents the prototype that originated from a patient with cancer, was rare worldwide except in Asia.

Conclusions. HPV-58 can be classified into 4 lineages that show some degree of ethnogeographic predilection in distribution. The evolutionary, epidemiological, and pathological characteristics of these lineages warrant further study.

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Human papillomavirus (HPV) infection is a necessary, although insufficient, cause of cervical cancer, which is still the second leading cancer to affect women worldwide [1]. Two prophylactic vaccines (Cervarix and Gardasil) targeting the 2 most prevalent high-risk HPV types (HPV types 16 and 18) found in cervical cancer are available [2, 3]. These vaccines are expected to prevent ~70% of cervical cancers worldwide [4]. There remain at least 13 high-risk HPV types not targeted by the

current vaccines that account for the remaining cervical cancers [5]. Globally, HPV types 31, 33, and 45 form the second group and HPV types 35, 52, and 58 form the third group in the ranking of cancer association [6]. However, the disease impact associated with these other HPV types shows considerable geographical variation. As shown in a meta-analysis, HPV type 58 (HPV-58) was found in 3.3% of cervical cancers globally and 5.6% of cervical cancers in Asia, whereas the prevalence in high-grade cervical intraepithelial lesions was 7.0% globally and 12.2% in Asia [4]. Studies from East Asian populations have reported an even higher rate. For instance, HPV-58 was detected in 26% of cervical squamous cell carcinoma in Shanghai [7], 16% in South Korea [8], 10% in Hong Kong [9] and Taiwan [10], and 8% in Japan [11]; and HPV-58 ranked third in cervical cancer cases from Asia overall [12]. Although HPV-58 may not play an etiological role in all these cases, as some of them are found in coinfections with other high-risk types, the disease impact conferred by HPV-58, especially in East Asian populations, cannot be neglected. The reason for a geographical or ethnical predilection of HPV-58-associated cervical neoplasia is not fully understood. Previous studies have suggested that host genetic factors and the circulation of variants with higher oncogenicity could play a role [13, 14]. To date, information on sequence variability of HPV-58 is very limited. This study was conducted to elucidate the phylogenetic relationship between HPV-58 variants collected worldwide to establish a classification system that will facilitate further study on the oncogenic potential of HPV-58.

MATERIALS AND METHODS

Study Samples

Cervical, vaginal, or anal samples were collected by study collaborators. Those samples that had tested positive for HPV-58 were transferred to a central laboratory for sequence analysis. The quality of DNA was assessed by amplifying a 1039-bp fragment of the L1 region, and the identity of HPV-58 was ascertained by demonstrating a nucleotide sequence similarity of >90% compared with the corresponding L1 fragment of the prototype (GenBank accession no. NC_001443). Altogether, 401 samples collected from 15 geographical locations had sufficient DNA quality for sequencing of the whole length of the E6, E7, E2 (containing E4), E5, L1, and LCR regions (Table 1). All samples except 37 anal swab specimens from men in the United States were cervical, vaginal scrape, swab, or tissue specimens from women. The distribution of cervical pathology status is shown in Table 1.

Nucleotide Sequencing

The whole lengths of E6, E7, E2 (containing E4), E5, L1, and LCR were amplified separately by polymerase chain reaction (PCR) using primers designed on the basis of the prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443; primer sequences are shown in the Supplemental Data). Briefly, the PCR was conducted in a 50-μL reaction mix containing 4 μL of extracted DNA, 200 μmol/L deoxynucleotide triphosphates, forward and reverse primers at .25 μmol/L each, and 1.25 U of HotStarTaq Plus polymerase (Qiagen). The cycling conditions were as follows: activation of polymerase at 95°C for 5 min, 40 cycles of denaturation at 94°C for 50 s, annealing at 56°C–60°C for 50 s, and extension at 72°C for 50–70 s, followed by a final extension at 72°C for 8 min. Amplification was visualized by agarose gel electrophoresis. Samples with insufficient amplification product for sequencing were subjected to another round of PCR using the nested primers.

PCR products were purified by Microspin S-400 columns (GE Healthcare). Ten microliters of the purified PCR products were mixed with 2 μL of BigDye Terminator sequencing reaction mix (version 3.1; Applied Biosystems), 3 μL of 5× sequencing buffer, and 3.2 pmol of the sequencing primer; and made up to a final volume of 20 μL according to the manufacturer's instructions. The cycling conditions for the labeling PCR were 25 cycles at 95°C for 15 s, 50°C for 15 s, and 65°C for 75 s. Fluorescence-labeled PCR products were purified with DyeEx (Qiagen) and run on an ABI 3130 automated sequence analyzer (Applied Biosystems). Sequence data were obtained from both directions and analyzed with SeqScape software (version 2.5; Applied Biosystems). Mutations that occurred only once were confirmed by repeating from the original sample.

Naming of Variants and Phylogenetic Tree Construction

Variant sequences were named WW for "worldwide," followed by a number according to its prevalence as found in this study. The concatenated nucleotide sequences from the 5 complete open reading frames (ORFs; E6, E7, E2, E5, and L1) and the LCR region of individual variants were used for phylogenetic tree construction. The tree construction processes were repeated for each genomic region to identify the most informative surrogate region for lineage classification of HPV-58 variants.

Maximum-likelihood trees were constructed using the program PAUP* (version 4.0b10) [15]. Modeltest (version 3.7) [16] was used to identify the best evolutionary model. A neighbor-joining tree was constructed as a starting tree, followed by a maximum-likelihood tree using the subtree pruning and regrafting (SPR) search approach. The data were bootstrap resampled 1,000 times. To verify the tree topologies observed from maximum-likelihood trees, the program MrBayes (version 3.1.2) [17] was used for Bayesian tree construction, with the nucleotide substitution model set according to the Modeltest results. The Markov chain Monte Carlo analysis was run for 5,000,000 generations with trees sampled at every 1,000 generations. A burn-in rate of 25% was used in summarizing the data. The trees were displayed with Figtree (version 1.1.2; http://tree.bio.ed.ac.uk/software).

Table 1. Distribution of Study Samples According to the Source of Collection and Cervical Pathology Status

Region, country or city	Total no. of specimens	No. of specimens of each cervical pathology status					
		Normal	ASCUS	LGSIL	HGSIL	Carcinoma	Unknown
Africa	...	...	...	...	...	...	...
Zimbabwe[a]	69	0	0	0	0	0	69
Americas	63	6	1	6	10	2	1[b]
Canada	10	2	0	4	4	0	0
United States	37	...[c]	...[c]	...[c]	...[c]	...[c]	...[c]
Mexico	2	0	0	0	1	1	0
Argentina	6	3	1	0	0	1	1
Brazil	3	1	0	1	1	0	0
Honduras	5	0	0	1	4	0	0
Asia	238	57	23	57	66	31	4
Mainland China	3	0	0	0	0	0	3
Hong Kong	90	17	0	15	41	17	0
Taiwan	6	0	0	0	0	6	0
South Korea	119	30	23	40	22	4	0
Japan	14	9	0	2	1	1	1
Thailand	6	1	0	0	2	3	0
Europe	31	16	1	9	3	0	2
United Kingdom	14	6	0	6	2	0	0
Italy	17	10	1	3	1	0	2
Total	401	83	26	68	72	34	81[b]

NOTE. ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HGSIL, high-grade squamous intraepithelial lesions (including CIN2, CIN3, and severe dysplasia); LGSIL, low-grade squamous intraepithelial lesions (including CIN1 and mild dysplasia);

[a] Cervical or vaginal swabs.

[b] Not including 37 anal swab samples.

[c] Anal swab samples.

Geographical Distribution of Variant Lineages

The rate of detection of each variant lineage was compared among the 4 regions by use of a Pearson χ^2 test. When a significant difference was obtained between regions (defined as $P < .05$), detection rates between regions were compared 2 by 2 with a Fisher exact test, for a total of 6 comparisons. The level of significance for 6 possible comparisons was then set at .008 according to the Bonferroni correction.

The distribution of variant lineages among anal swabs that were collected from men at a single center in the United States was compared with that among cervical samples collected from other parts of the Americas by use of a Pearson χ^2 test or a Fisher exact test as appropriate. The association between variant lineage and cervical pathology status was assessed by a Fisher exact test. Two-tailed P values of $<.05$ were regarded as significant.

RESULTS

Lineage Classification

Altogether, 268 unique concatenated E6-E7-E2-E5-L1-LCR nucleotide sequences of HPV-58 variants were assembled. The lengths ranged from 4416 bp to 4462 bp, accounting for ~57% of the whole viral genome. Since none of the assembled sequences was identical to the prototype, the sequence available at GenBank (GenBank accession no. NC_001443) was used to assemble a concatenated prototype sequence to serve as a reference. The maximum-likelihood tree revealed 4 clusters (Figure 1), and the topology was same as that observed from the Bayesian tree.

Among all the genomic regions examined, L1 and LCR displayed tree topologies that most closely resembled that of the concatenated E6-E7-E2-E5-L1-LCR sequences. The L1 and LCR regions were considered to be most informative surrogate regions for phylogenetic grouping of HPV-58 variants when the full genome sequence is not available. The lineage containing the prototype was assigned as lineage A, and the others were arbitrarily designated as lineages B, C, and D. Figures 2A and 2B show the maximum-likelihood trees of the L1 and LCR sequences, which revealed topologies that were same as those observed from the Bayesian trees. The 2 most prevalent L1 variants, L1_WW001 and L1_WW002, were found in 26.7% and 11.7% of samples, respectively; whereas the L1_WW054 variant, found in .2% of samples, is the prototype. The 2 most prevalent LCR variants, LCR_WW001 and LCR_WW002, were found in 20.8% and 11.5% of isolates, respectively. The variant LCR_WW009, which was found in 3.6% of isolates, is

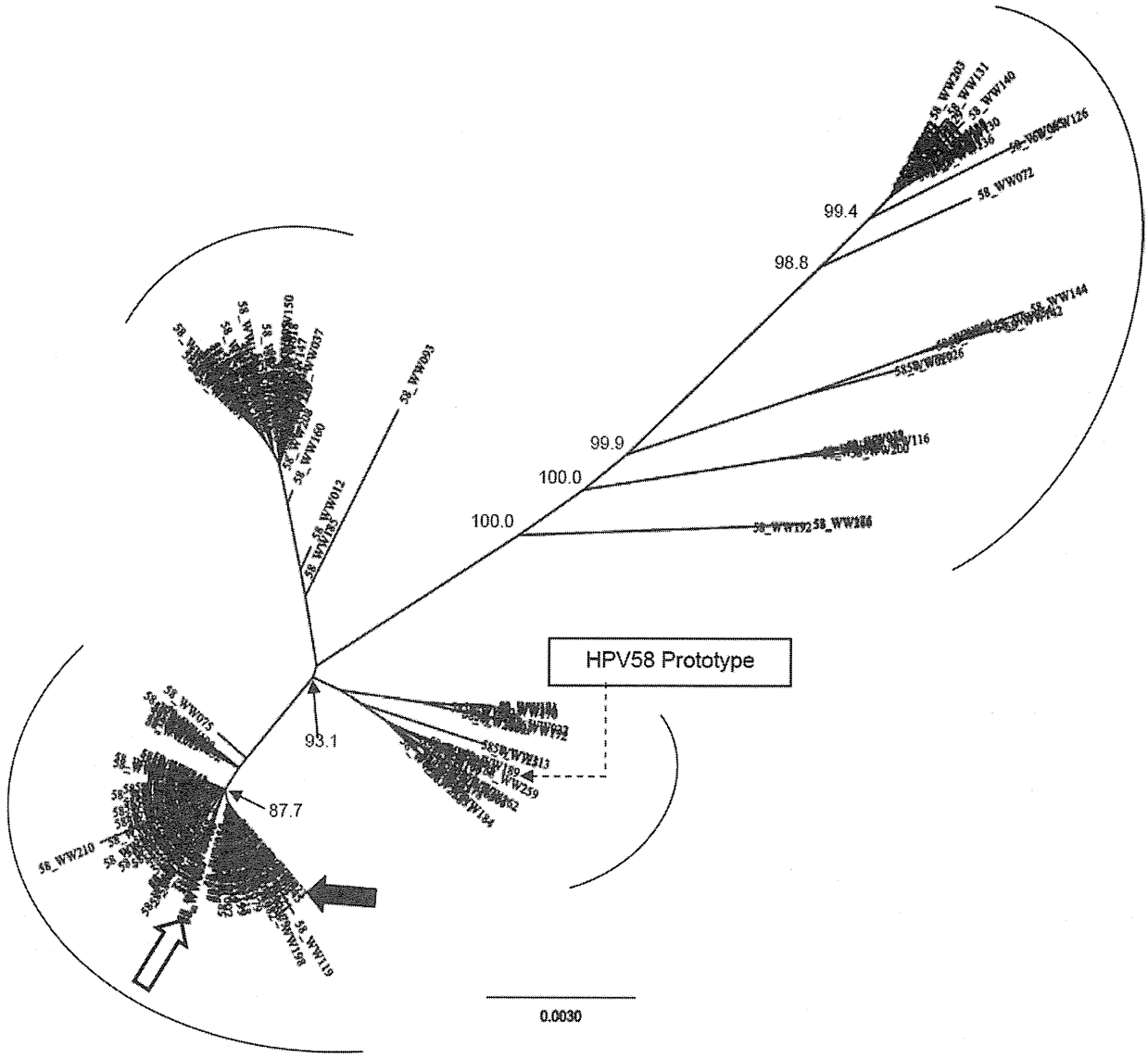


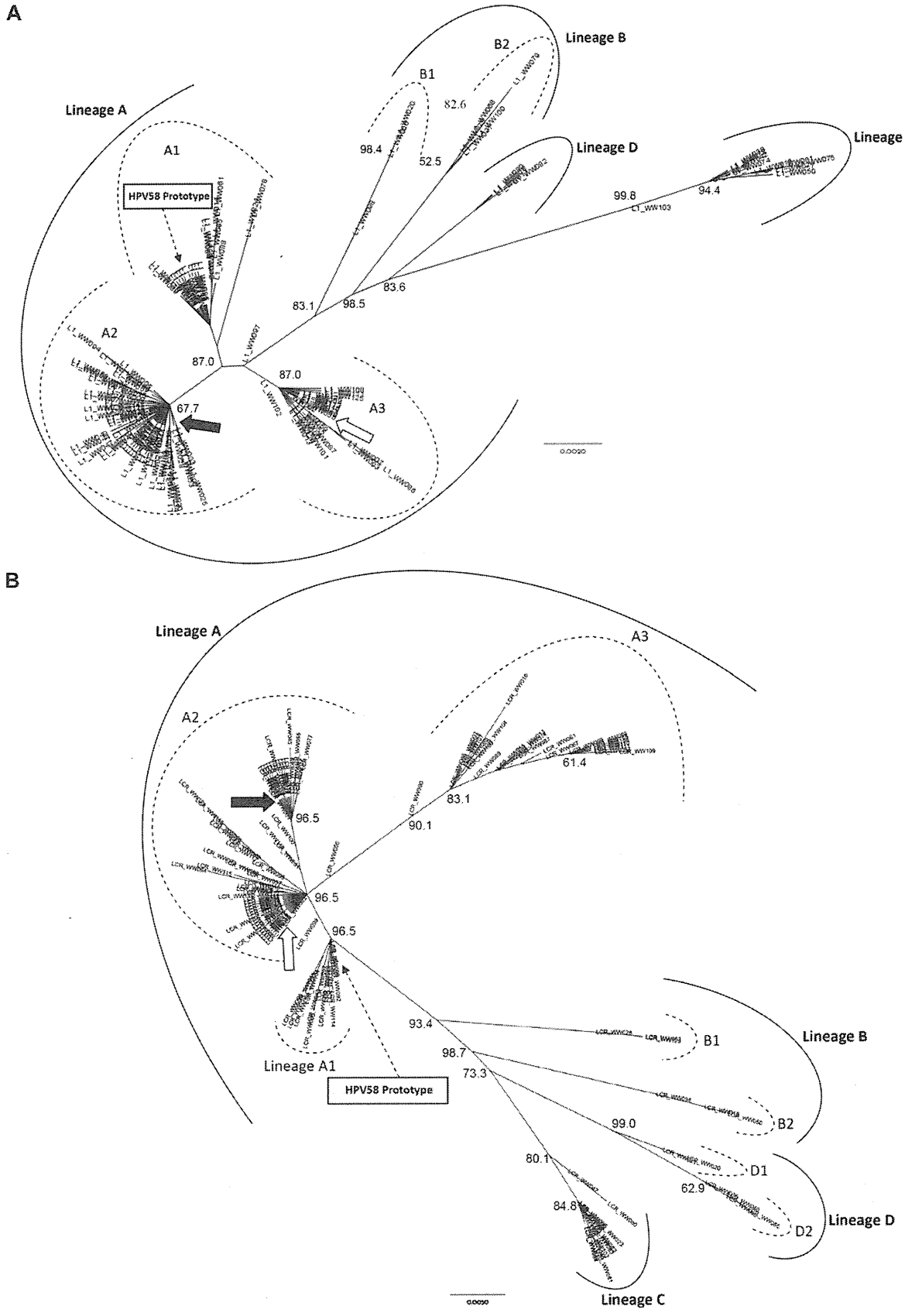
Figure 1. Phylogenetic tree constructed from concatenated E6-E7-E2-E5-L1-LCR nucleotide sequences of 268 human papillomavirus type 58 (HPV-58) Variants. The maximum-likelihood tree was constructed with the PAUP* program (version 4.0) using the GTR+I+G model for nucleotide substitution. Bootstrap values of >70% generated by 1,000 resamplings are shown. The length of the scale bar represents .003 substitutions per nucleotide position. The position of the HPV-58 prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443) is indicated. The 2 most prevalent variant sequences, WW_001 and WW_002, are marked with a black arrow and a white arrow, respectively.

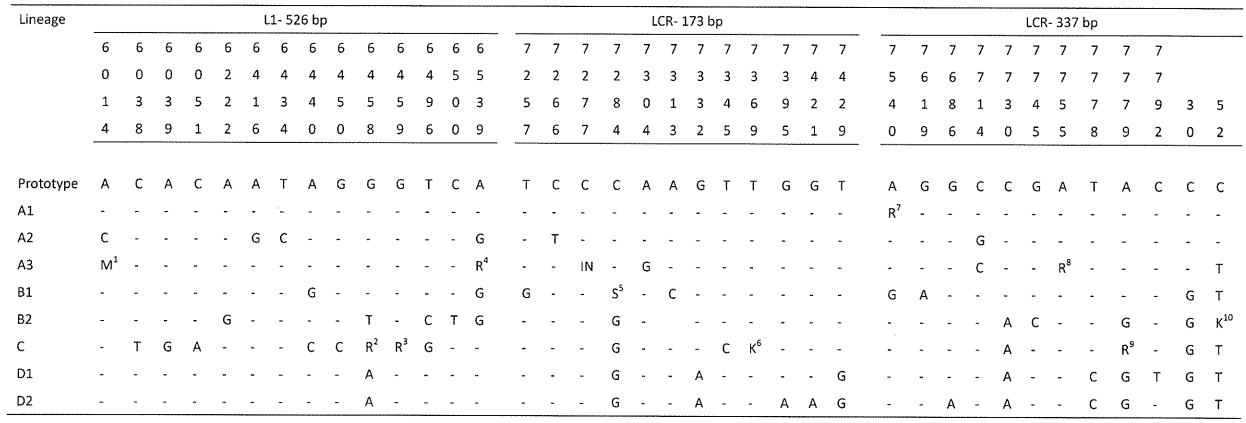
the prototype. None of the samples studied were found to harbor a mixture of variants.

The sequence variations of the L1 and LCR regions among different lineages were examined to identify shortest fragment or fragments that contain sequence signatures unique for each lineage. As a result, 3 fragments were found to be the best surrogate targets for lineage identification, including a 526-bp L1 fragment that corresponds to nucleotide position 6014–6539, a 173-bp LCR fragment at nucleotides 7257–7429, and another 337-bp LCR fragment at nucleotides 7540–52. The sequence variations at key positions are shown in Figure 3.

Geographical Distribution of HPV-58 Lineages

Figure 4 shows the distribution of HPV-58 lineages in each geographical location. Lineage A was the most prevalent lineage found worldwide (86.0% of isolates), as well as in each region (49.3%–95.8% of isolates). The prevalence of lineage A in Africa (49.3% of isolates) was significantly lower than in other regions (85.7%–95.8% of isolates; $P < .001$ for each comparison), whereas lineage A was significantly more frequent in Asia than in the Americas ($P = .007$). Lineage B was found in 2.5% of the isolates collected worldwide, ranging from none in Europe to 3.2% in the Americas. The number of lineage B isolates was too





Lineage	L1- 526 bp														LCR- 173 bp												LCR- 337 bp										
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7		
	0	0	0	0	2	4	4	4	4	4	4	4	5	5	2	2	2	2	3	3	3	3	3	3	4	4	5	6	6	7	7	7	7	7	7	7	
	1	3	3	5	2	1	3	4	5	5	5	9	0	3	5	6	7	8	0	1	3	4	6	9	2	2	4	1	8	1	3	4	5	7	7	9	3	5
	4	8	9	1	2	6	4	0	0	8	9	6	0	9	7	6	7	4	4	3	2	5	9	5	1	9	0	9	6	4	0	5	5	8	9	2	0	2
Prototype	A	C	A	C	A	A	T	A	G	G	G	T	C	A	T	C	C	C	A	A	G	T	T	G	G	T	A	G	G	C	C	G	A	T	A	C	C	C
A1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R^7	-	-	-	-	-	-	-	-	-	-	-
A2	C	-	-	-	-	G	C	-	-	-	-	-	-	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-
A3	M^1	-	-	-	-	-	-	-	-	-	-	-	-	R^4	-	-	IN	-	G	-	-	-	-	-	-	-	-	-	-	C	-	-	R^8	-	-	-	-	T
B1	-	-	-	-	-	-	-	G	-	-	-	-	-	G	G	-	-	S^5	-	C	-	-	-	-	-	-	G	A	-	-	-	-	-	-	-	-	G	T
B2	-	-	-	-	G	-	-	-	-	T	-	C	T	G	-	-	-	G	-		-	-	-	-	-	-	-	-	-	-	A	C	-	-	G	-	G	K^{10}
C	-	T	G	A	-	-	-	C	C	R^2	R^3	G	-	-	-	-	-	G	-	-	-	C	K^6	-	-	-	-	-	-	-	A	-	-	-	R^9	-	G	T
D1	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	G	-	-	A	-	-	-	-	G	-	-	-	-	A	-	-	C	G	T	G	T
D2	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	G	-	-	A	-	-	A	A	G	-	-	A	-	A	-	-	C	G	-	G	T

Figure 3. Signature sequences for human papillomavirus type 58 (HPV-58) lineage identification. Numbers refer to nucleotide position of the HPV-58 prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443). A dash represents the same nucleotide as in the prototype. IN, insertion of 12 bp. For M^1, 91% of isolates have C and 9% have A; for R^2, 54.5% have G and 45.5% have A; for R^3, 91% have A and 9% have G; for R^4, 96% have G and 4% A; for S^5, 67% have C and 33% have G; for K^6, 86% have G and 14% have T; for R^7, 78.6% have A and 21.4% have G; for R^8, 96% have G and 4% have A; for R^9, 86% have G and 14% have A; and for K^{10}, 67% have T and 33% have G.

few for statistical analysis. Lineage C was found in 9.2% of isolates collected worldwide and was found significantly more frequently in Africa than in Asia, the Americas, or Europe ($P \leq .001$ for each comparison). Lineage D has a worldwide prevalence of 2.2% and seemed to be more frequent in Africa (8.7%). However, the number of lineage D isolates was too few for statistical analysis.

Since lineage A was the most prevalent lineage identified, a subgroup analysis was performed for the distribution of sublineages A1, A2, and A3. Sublineage A2 was the most frequently detected sublineage, accounting for 62.3% of lineage A isolates collected worldwide and dominating in Africa (94.1%), the Americas (87.0%), and Europe (93.1%). In contrast, sublineages A1, A2, and A3 were more evenly distributed in Asia, accounting for 16.2%, 47.8%, and 36.0%, respectively, of lineage A isolates found in this region. As a result, the proportion of lineage A isolates belonging to sublineage A2 was significantly lower in Asia than in other regions ($P < .001$), whereas the proportion belonging to sublineage A3 was significantly higher in Asia than in other regions ($P < .001$).

Of the 37 anal swabs collected from men in the United States, 30 (81.1%) were lineage A, 1 (2.7%) was lineage B, 5 (16.7%) were lineage C, and 1 (.3%) was lineage D. The proportion of each lineage among these samples was not significantly different ($P = .06$–$.4$) from that of samples collected from women in the Americas.

Lineage Distribution and Cervical Pathology

In Asia, lineage A accounted for 94.8% of high-grade squamous intraepithelial lesion (HGSIL) and carcinoma samples and 96.4% of samples of normal cytology, low-grade squamous intraepithelial lesion (LGSIL), and atypical squamous cells of undetermined significance (ASCUS); no significant difference in the distribution of lineage A compared with non-A lineages was found ($P = .745$). Similarly, lineage A accounted for the majority of HGSIL/carcinoma samples in Hong Kong (94.8%) and South Korea (92.3%), and no significant association between lineage and lesion severity was found ($P = 1.0$ for Hong Kong; $P = .120$ for South Korea). The number of samples available from other regions was too few for a similar statistical analysis.

DISCUSSION

Analysis of intratypic sequence variation of HPV can provide important information for the design of diagnostic tools, development of vaccines, identification of molecular markers for epidemiological studies, elucidation of implications of sequence variation on biological and pathological properties, and

Figure 2. Phylogenetic trees of L1 and LCR sequences of human papillomavirus type 58 (HPV-58) variants. The maximum-likelihood tree was constructed with the PAUP* program (version 4.0) using the GTR+I+G model for nucleotide substitution. Bootstrap values of key positions generated with resampling 1,000 times are shown. The length of the scale bar represents the number of substitutions per nucleotide position. The position of the prototype (http://www.ncbi.nlm.nih.gov/genbank accession no. NC_001443) is indicated. The 2 most prevalent variant sequences, WW_001 and WW_002, are marked with black and white arrows, respectively. *A*, L1 open reading frame of 121 variants (HM639317–HM639717). *B*, LCR open reading frame of 123 variants (HQ338950–HQ339350).