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ORIGINAL INVESTIGATION

No evidence of chikungunya virus and antibodies shortly before the outbreak on Sri Lanka

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Abstract A massive outbreak of chikungunya disease occurred on Sri Lanka in 2006. Reasons for the explosive nature of the epidemic are being intensively discussed. According to recognised and anecdotal concepts, absence of human population immunity against chikungunya virus (CHIKV) might have supported virus amplification. However, formal proof of concept is lacking. This study determined the prevalence of anti-CHIKV IgG antibodies as well as CHIKV RNA shortly before the outbreak. Two hundred and six human sera were collected from patients with acute febrile illness in 2004/2005. Validated indirect immunofluorescence and real-time RT-PCR assays for dengue as well as CHIKV were employed. Laboratory evidence of dengue virus infection was seen in 67% of patients, indicating virus activity and exposure to *Aedes* spp. vectors. These vectors are the same as for chikungunya. However, no evidence of acute or previous chikungunya infection could be demonstrated in the same cohort. This study gives formal evidence that the absence of human population immunity correlated with a large chikungunya epidemic.

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Introduction

Chikungunya disease is caused by chikungunya virus (CHIKV), an arthropod-borne Alphavirus in the *Togaviridae* family. Transmission occurs via various *Aedes* species. Main symptoms in humans are fever, skin rash and severe arthralgia [1]. Arthralgias can be long-lasting and incapacitating with significant impact on individual as well as public health [2]. In 2005 and 2006 the western Indian Ocean region was hit by an extraordinarily large chikungunya disease epidemic. It reached India by the end of 2005, infecting more than 1.3 million people [3]. Incidence on neighbouring Sri Lanka started in October 2006 [4, 5]. In a total population of about 20 million, 37,667 suspected cases according to a strict case definition were notified in the ensuing outbreak [5].

Outside recurring epidemics, CHIKV spills into the human population accidentally from sylvatic transmission cycles established between vectors and diverse amplifying hosts (mammals including primates, sheep, rodents, bats; as well as birds) [6]. In contrast, urban transmission cycles are assumed for large recurrent outbreaks in Asia [6]. Patterns of recurrence of these outbreaks are unpredictable and can last up to 32 years as documented in India [7].

Different reasons for the explosive nature of the current outbreak have been discussed. Recent studies demonstrated that an A226V mutation in the CHIKV E1 glycoprotein confers an advantage in vector competence in *Ae. albopictus* but not in *Ae. aegypti* [8, 9]. Critically, this mutation occurred independently in different recent outbreak locations where *Ae. albopictus* but not *Ae. aegypti* predominated (La Reunion, Cameroon, Gabon) [10, 11]. On the

other hand, India with its mixed vector population experienced one of the most severe outbreaks of urban chikungunya disease in 2005/2006, despite absence of the mutation [3, 11].

Another explanation for the severity of the outbreak would be the absence of pre-existing antibodies in the population. The establishment of urban transmission theoretically requires a broadly susceptible human population that is exposed to competent vectors, thus amplifying the virus and transmitting it back to mosquitoes. This might require that a sufficient fraction of people have been born after the last epidemic. Unfortunately, information on the prior immunity level of vector-exposed human populations outside epidemics is not sufficiently documented in the literature [12].

In order to determine underlying CHIKV activity prior to the 2006/2007 epidemic on Sri Lanka, patients with likely exposure to insect vectors and compatible clinical symptoms were examined in this study. Since dengue virus (DENV) is co-endemic on Sri Lanka [13], current or recent DENV infection served as a surrogate for vector exposure. This study demonstrated that a lack of human herd immunity might indeed have contributed to the recent severe chikungunya epidemic on Sri Lanka.

Methods

Patients

Sampling took place from September 2004 to April 2005; 206 patients were seen at Peradeniya University Hospital in Kandy, Sri Lanka, reporting acute febrile illness. All patients stemmed from and lived in the Central province. Median age was 31 years (range 13–76 years). Eighty-six (42%) patients were female, 120 (58%) were male. 152 patients (74%) presented with a rash, another 152 with myalgias. Each individual provided one serum sample. Median reported duration of fever before drawing of blood was 4 days (range 1–14 days). Fever was measured and documented in 65/206 (32%) patients upon admission. In the remaining patients fever was not measured but confirmed upon clinical examination. Written informed consent was obtained from all patients. The study was approved by the committee on research and ethical review of the University of Peradeniya.

Dengue virus testing

All samples were screened on-site for DENV specific IgM antibodies by μ-capture enzyme immunoassay (EIA; Panbio, Sinnamon Park, QLD, Australia) as well as for DENV-specific IgG antibodies by EIA (Panbio) as recommended by the manufacturer. In Germany, all samples were tested for DENV RNA by real-time RT-PCR targeting the viral 3′-noncoding region [14]. Viral RNA was prepared from serum by means of a viral RNA mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The same RNA preparations were used for CHIKV real-time RT-PCR (see below). Laboratory classification of cases of acute DENV infection was based on the detection of either IgM or RNA but not on rise of IgG titre, as only one serum per patient was available.

Chikungunya virus testing

Independent of DENV status, all 206 samples were tested for anti-CHIKV IgG antibodies by a validated immunofluorescence assay [15, 16]. In brief, CHIKV strain S27 was grown on Vero cells at a multiplicity of infection of 0.5. After 24 h cells were spread on slides, air-dried and fixed in ice-cold acetone. Hereafter serum samples were incubated on the fixed cells for 1 h. A fluorescein isothiocyanate (Sifin, Berlin, Germany) labelled anti-human IgG antibody was used for detection of antibodies. To detect acute cases a validated CHIKV real-time RT-PCR targeting the *nsp1* gene was conducted as described [16]. IgM testing was not done because in our own previous studies we found that no acute cases were missed when IgG and PCR testing were used in combination [16].

A fragment of the E1 gene of a CHIKV strain derived directly from one German tourist plasma was sequenced on a CEQ 8000 Genetic Analysis System (Beckman-Coulter, Krefeld, Germany) as described [16].

Results

Laboratory results in 206 patients are summarised in Table 1. One hundred and thirty-nine of 206 (67%) patients showed laboratory evidence of acute or prior DENV infection (Table 1). Ninety-five (46.1%) patients in total had anti-DENV IgG antibodies. Seventy-eight (37.9%) had evidence of acute DENV infection, comprising 30 (46%) of those patients with measured fever upon admission and 48 (34%) of those in whom fever was only documented on grounds of history and clinical examination.

Neither anti-CHIKV IgG antibodies nor CHIKV RNA was detected in any patient, including 128 patients without evidence of acute DENV infection. Ninety-seven (76%) of these had myalgia, a lead symptom of chikungunya disease. Differences between acute DENV and CHIKV findings were highly significant (Chi-square test, $P < 0.0001$).

The outbreak in Sri Lanka can be temporally and spatially linked with that in India. However, because viruses from the Sri Lanka outbreak have not been characterised

Table 1 Laboratory findings

	No. (%) of patients positive by laboratory test			
	Acute infection			Past infection
	IgM only	IgM/IgG	RT-PCR only	IgG only
Dengue virus				
Total (n = 206)	40 (19)	34 (17)	4 (2)	61 (30)
Fever objectified[a] (n = 65)	18 (28)	11 (17)	1 (2)	14 (22)
Fever documented[b] (n = 141)	22 (16)	23 (16)	3 (2)	47 (33)
Chikungunya virus				
Total (n = 206)	0	0	0	0

[a] Exact temperature taken upon admission

[b] Exact temperature not taken, but fever was documented upon clinical examination and history

yet, we amplified virus from a German tourist returning from Sri Lanka in December 2006 and sequenced the E1 gene region [16]. It had Alanin at position 226 of E1, indicating absence of the mutation critical for enhanced vector competence in *Ae. albopictus*. Over a sequence of 648 bp, this virus was 100% identical with sequences from viruses isolated in 2006 from Karnataka (strain IND-06-KA15/EF027135 [3]) and Maharashtra (strain IND-06-MH2/EF027136 [3]), also showing the 226A genotype.

Discussion

Sri Lanka provides an ecological situation similar to South India, albeit in a more circumscript geographic setting. In analogy with India, it has been shown that in Sri Lanka *Ae. aegypti* and *Ae. albopictus* prevail at approximately equal ratios [17]. With regard to vector population and vector competence of the virus, both countries therefore must have had similar predisposing conditions before both experienced large outbreaks of chikungunya disease.

Anti-CHIKV IgG antibodies persist for prolonged times and correlate with virus clearance in humans [16, 18]. Serotypic heterogeneity between strains of CHIKV is unknown, and pre-existing IgG probably prevents infection and virus amplification in humans and primates [19]. Our patients had high rates of past or acute DENV infection, confirming that they were indeed exposed to either *Ae. aegypti*, *Ae. albopictus*, or both. With good confidence, absence of both CHIKV RNA and antibodies in these patients indicates absence of recent CHIKV activity in the Central Province of Sri Lanka. Interestingly, Peiris et al. have demonstrated 15 years ago the absence of anti-CHIKV antibodies in macaques in Polonnaruwa/Sri Lanka, about 100 km away from our sampling site [20]. Similar to humans in our study, these same primates had high rates of anti-DENV antibodies. Sri Lanka experienced the last severe epidemic of chikungunya disease in 1964/1965 [21]. Little or no activity was reported in the 1970/1980s [22].

Taken together, these data identify underlying conditions facilitating the 2006/2007 epidemic. When the virus was introduced from India prior to the 2006 epidemic, it probably had not acquired a genetic advantage regarding vector competence. Most likely, humans in Sri Lanka were widely susceptible to CHIKV infection, after a period of >40 years of low or absent virus activity. Probably they acted as an efficient reservoir for urban virus amplification. In addition to the important role of a new virus variant in recent CHIKV outbreaks, the causative relationship between human population immunity and urban virus transmission is emphasised. It has to be discussed whether available CHIKV vaccines [18, 23, 24], should be administered in populated centres of Asia in periods of low CHIKV activity.

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Conflict of interest statement None declared.

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