Infection with chikungunya virus in Italy: an outbreak in a temperate region

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Summary

Background Chikungunya virus (CHIKV), which is transmitted by *Aedes* spp mosquitoes, has recently caused several outbreaks on islands in the Indian Ocean and on the Indian subcontinent. We report on an outbreak in Italy.

Methods After reports of a large number of cases of febrile illness of unknown origin in two contiguous villages in northeastern Italy, an outbreak investigation was done to identify the primary source of infection and modes of transmission. An active surveillance system was also implemented. The clinical case definition was presentation with fever and joint pain. Blood samples were gathered and analysed by PCR and serological assays to identify the causal agent. Locally captured mosquitoes were also tested by PCR. Phylogenetic analysis of the CHIKV E1 region was done.

Findings Analysis of samples from human beings and from mosquitoes showed that the outbreak was caused by CHIKV. We identified 205 cases of infection with CHIKV between July 4 and Sept 27, 2007. The presumed index case was a man from India who developed symptoms while visiting relatives in one of the villages. Phylogenetic analysis showed a high similarity between the strains found in Italy and those identified during an earlier outbreak on islands in the Indian Ocean. The disease was fairly mild in nearly all cases, with only one reported death.

Interpretation This outbreak of CHIKV disease in a non-tropical area was to some extent unexpected and emphasises the need for preparedness and response to emerging infectious threats in the era of globalisation.

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus transmitted to human beings by *Aedes* spp mosquitoes. After the isolation of the virus in Tanzania in 1953,[1] sporadic cases and a number of outbreaks of infection with CHIKV have been reported in several African countries, on the Indian subcontinent, and in southeast Asia.[2] In the past few years, a series of outbreaks have been reported over a large geographical area that includes African islands in the Indian Ocean and the Indian subcontinent. The first of the outbreaks occurred in Kenya in 2004, followed by outbreaks on the Comoros Islands, the island of La Réunion, and other islands in the southwest Indian Ocean in early 2005, and by a large outbreak in India in 2005–06.[3,4] According to the molecular analysis of the strains isolated on islands in the Indian Ocean and in India, the epidemic was caused by a variant of the central/east African genotype of CHIKV.[5]

During the outbreak on islands in the Indian Ocean, a large number of travellers from industrialised countries with a temperate climate became infected with CHIKV and were still infected on returning home.[6–9] In some of these industrialised countries, *Aedes albopictus*—a vector of CHIKV—was introduced a number of years ago and is now widespread,[10] with an especially high population density in Italy.[11] This situation is particularly threatening because it has been suggested that the strain of CHIKV in the Indian Ocean has better adapted to *A albopictus* than it has to other *Aedes* spp.[4] Nonetheless, to date, no outbreaks due to the local transmission of CHIKV have been reported in these countries. Here, we report on a large outbreak of CHIKV infection that occurred in two neighbouring villages in Italy.[12]

Methods

Patients

In July and August, 2007, the local health unit of the province of Ravenna (region of Emilia Romagna, northeastern Italy) detected an unusually high number of cases of febrile illness in Castiglione di Cervia and Castiglione di Ravenna, two small villages divided by a river. In the second week of August, the local health unit implemented an active surveillance system to identify, both prospectively and retrospectively, all individuals with febrile illness, on the basis of reports provided by general practitioners and hospital emergency units. Patient data were collected with a standardised questionnaire and included age, sex, place of residence, countries visited, travel dates, and date of onset of symptoms. In late August, an outbreak investigation was done to identify the agent and the source of the infection and to better understand the dynamics of the epidemic of febrile illness.

Early in the outbreak investigation, infection with CHIKV was suspected because of clinical symptoms and the fact that the first patient with febrile illness was a man from a country affected by an outbreak. Furthermore, the presence of *A albopictus* in the area was known. A case of CHIKV infection was defined as the presence of

high fever (>38·5°C) and joint pain and living in, or having visited, one of the two villages; for this definition, laboratory confirmation was not required. Individuals with fever but no joint pain and those with these symptoms who did not live in the villages or who had not visited them were deemed to be cases only if laboratory confirmation was obtained (ie, positivity to either haemagglutination inhibition or PCR).

Procedures

Blood and serum samples were stored at –80°C before being tested. Samples were tested for antibodies to CHIKV by haemagglutination inhibition and initially confirmed by plaque reduction neutralisation assays in 12 cases who were haemagglutination inhibition-positive; both tests were done at the Istituto Superiore di Sanità, Rome.[11] Thereafter, only the haemagglutination inhibition test was used. Samples were also tested for antibodies to dengue virus and yellow fever, also with the haemagglutination inhibition test.

To detect the presence of viral genomic RNA in human samples, real-time RT-PCR targeting the *nsp1* gene of CHIKV was done. The assay was based on the Qiagen One Step RT-PCR kit, and a 25 µL reaction volume included 3 µL RNA extract (Qiagen Viral RNA Mini kit), 40 ng/µL bovine serum albumin, 400 µmol/L of each dNTP, 600 nmol/L CHIKV sense (tgatcccgactcaaccatcct), 600 nmol/L CHIKV anti-sense (ggcaaacgcagtggtacttcct) primers, and 200 nmol/L probe ChikP (FAM-tccgac-atcatcctccttgctggc-Black Hole Quencher 1). Amplification was done in a Roche Light Cycler (Indianopolis, IN, USA) and involved the following steps: 50°C for 30 min, 95°C for 15 min, and 45 repetitions of 95°C for 15 s then 58°C for 30 s.

PCR was also used to detect CHIKV in specimens of *A albopictus* that were captured locally during the outbreak. Total RNA was extracted from the supernatant of an homogenate of mosquitoes in minimal essential medium, using TRIzol LS (Invitrogen, Carlsbad, CA, USA)). The RNA was retrotranscribed to cDNA with SuperScript II (Invitrogen) and random primers. Two different PCR protocols were used on the same samples: an RT nested PCR[14] and a real-time PCR with Taqman probe.[15]

Two pairs of primers (CHIKV 10264F/CHIKV 11300R and CHIKV 10564F/CHIKV 11081R)[16] were used to

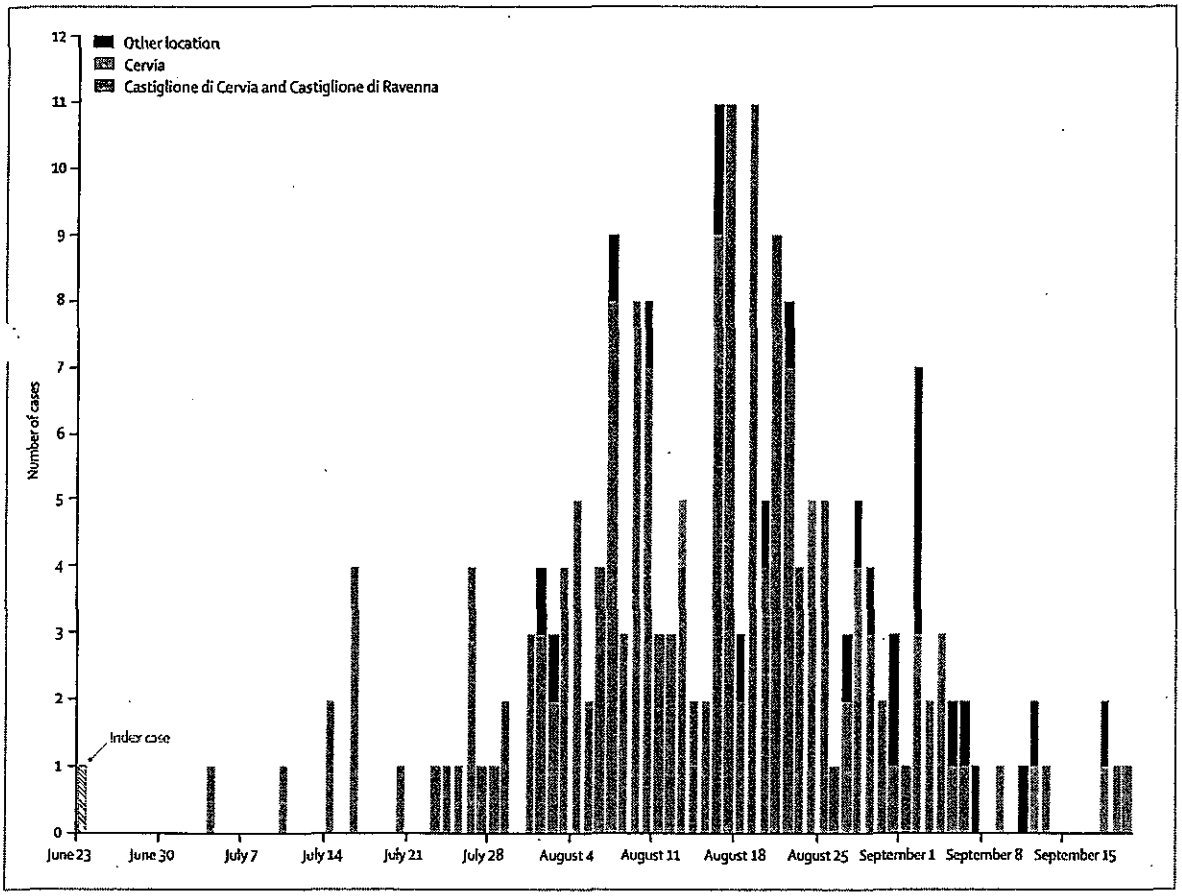

Figure 1: **Epidemic curve**
Distribution of dates of onset of symptoms for CHIKV cases by presumed place of infection (ie, Castiglione di Cervia and Castiglione di Ravenna, Cervia, or other/unknown location).

amplify part of the E1 gene directly from the extracted RNA from RT-PCR-positive samples. Nucleotide sequences were assembled with BioEdit software (version 7·0·9·0) and were aligned with Clustal W software (version 1.6). Phylogenetic analysis based on the available partial E1 gene sequences of CHIKV and tree reconstructions were done with MEGA version 4. For the construction of phylogenetic trees, the neighbour-joining algorithm and the Kimura two-parameter distance model were used. The reliability of the analysis was assessed by a bootstrap test with 1000 replications.

Statistical analysis

In the present analysis, we considered those cases identified between July 4 and Sept 27, 2007. The dates of the onset of symptoms of the cases were plotted to fit the epidemic curve. The frequency distribution of the cases' main characteristics and their signs and symptoms were calculated. Attack rates (both overall and stratified by age and sex) were calculated for the two villages that were affected. Risk ratios (RR) and their 95% CI were also estimated. Age-adjusted attack rates were also calculated separately by sex. The origin and spread of CHIKV cases in the two initially affected villages were mapped. For each case, the address of the individual and the date of the onset of symptoms were entered into Microsoft Access 2003 and linked to the locations on georeferenced maps in the geographic information system (ArcView 8·3, ESRI, Redlands, CA, USA).

Role of the funding source

There was no funding source for this study. All authors had full access to all the data. The corresponding author had final responsibility for the decision to submit for publication.

Results

205 cases of CHIKV infection occurred between July 4 to Sept 27, 2007, in Ravenna (figure 1). There were several waves of cases, with the number peaking in the third week of August. Up to the time of this peak, most cases had occurred in Castiglione di Cervia and Castiglione di Ravenna. Afterwards, and after the first mosquito control measures in the area that was mainly affected had been implemented (on Aug 18), a new wave of cases was observed, most of which occurred outside the two villages.

The distribution of cases by age, sex, residence, and place where the infection was presumably acquired is shown in table 1. The median age was 60 (range 1–95) years; 58 (1–92) years for male cases and 62 (3–95) years for female cases. Most patients reported that they lived in or had visited one of the two villages. The others were scattered throughout the province, although a cluster of 13 cases due to local transmission was reported in Cervia, a town of 8606 inhabitants located about 9 km from the villages. This cluster occurred in a restricted area of the town, near a public gathering place frequented by people coming from—or who had visited—Castiglione di Cervia and who had developed the disease.

	Number of cases (%)
Age (years)	
0–19	12 (6%)
20–39	26 (13%)
40–59	62 (30%)
60–70	78 (38%)
≥80	27 (13%)
Sex	
Male	99 (48%)
Female	106 (52%)
Presumed place of infection	
Castiglione di Cervia or Castiglione di Ravenna	171 (83%)
Cervia	13 (6%)
Other/unknown	21 (10%)
Classification of cases	
Laboratory confirmed	175 (85%)
Clinically defined (untested)	30 (15%)

Table 1: Demographic characteristics of the 205 individuals infected with CHIKV

The first identified case, a man of Indian origin living in Castiglione di Cervia, reported that he had not been abroad during the previous year. However, a relative of his, who had arrived in Italy on June 21 from Kerala, India (an area affected by the CHIKV epidemic), visited the man and became feverish on the afternoon of June 23, when he was in Castiglione di Cervia. A serum sample that had been collected in early September from this man, who was assumed to be the index case, showed high antibody titres against CHIKV (>1:1280). This individual was excluded from the further data analyses.

The spatial-temporal spread of CHIKV in the primarily affected area and the rest of the province is shown in figure 2. After the first cases, which occurred in Castiglione di Cervia, the infection spread both by contiguity, as an expansion of the primary cluster (figure 2 A, B, and C), and by jumping from place to place in both villages, with cases developing more than 2 km away from the primary cluster (figure 2 B and C). Sporadic cases and clusters occurring outside the villages are shown in figure 2 D.

The attack rate was 5·4% in Castiglione di Cervia (115 resident cases out of 2134 inhabitants) and 2·5% in Castiglione di Ravenna (46/1834). The attack rate did not differ between female and male individuals (4·5% of 81 females *vs* 4·0% of 80 males; RR 1·13, 95% CI 0·81–1·57). The rate of attack increased with age: 1·6% of 27 people under 40 years of age, 4·5% of 52 individuals aged 40–59 years, 7·0% of 57 aged 60–79 years, and 8·8% of 25 aged 80 years or older were affected (RR 2·78, 95% CI 1·75–4·39 for the 40–59 years age-group; 4·21,

Figure 2: **Geographical origin and spatial-temporal diffusion of CHIKV cases**
Number of cases in Castiglione di Cervia and Castiglione di Ravenna between days 0–15 (A), between days 0–45 (B), cumulatively (C), and in the province of Ravenna (D).

2·86–6·61 for the 60–79 years age-group; and 5·20, 3·08–8·83 for those aged 80 years or more, all relative to the under 40 years age-group; χ^2 for trend p<0·0001). There was no difference in attack rate between those aged 0–19 years and those aged 20–39 years (1·6% [10/631 individuals] *vs* 1·6% [17/1082]). The age-adjusted attack rates for male and female individuals were much the same (4·2% *vs* 4·1%).

The frequency of clinical symptoms is shown in table 2. All patients presented with high fever (median maximum temperature 39·5°C, 25–75th percentile 39–39·8°C), and most of them had pain in multiple joints. About half the cases developed skin rash, in some cases with itching. Clinical disease was mild and self-limiting in most cases. One 83-year-old man died, although this man had severe underlying conditions.

Laboratory confirmation was obtained for 175 cases: 32 were PCR-positive only; 135 were haemagglutination inhibition-positive only; and eight were positive for both PCR and haemagglutination inhibition. The median time between the onset of symptoms and obtaining positive results was 2 days for PCR (maximum 7 days) and 15 days for haemagglutination inhibition. 30 cases who met the clinical and epidemiological criteria

	Number of cases (%)
Fever	205 (100%)
Joint paint	199 (97%)
Fatigue	190 (93%)
Skin rash	106 (52%)
Headache	105 (51%)
Muscle pain	94 (46%)
Diarrhoea	48 (23%)
Itching	42 (20%)
Vomiting	40 (19%)
Photophobia	31 (15%)
Conjunctivitis	7 (3%)

†Mandatory in the case definition, ‡Not mandatory if diagnosis is laboratory confirmed.

Table 2: **Distribution of symptoms**

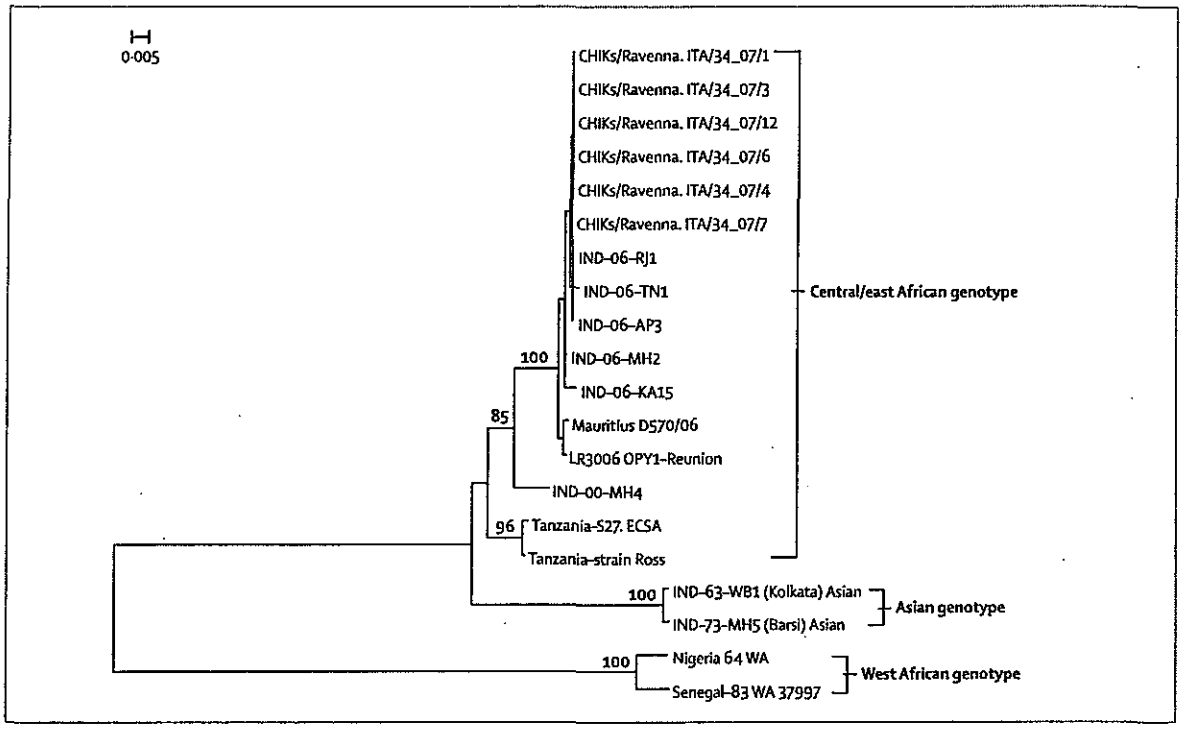

Figure 3: **Phylogenetic analysis of the partial nucleotide sequence (1011 nucleotides) of the E1 gene of CHIKV strains identified in Italy and in different parts of the world**

remained untested because no blood sample was available or because it was inadequate for testing (table 1).

CHIKV sequences were detected by PCR in *A albopictus* mosquitoes captured during the outbreak. Positive results were obtained from pools of 125 mosquitoes from Castiglione di Ravenna and and 90 from Castiglione di Cervia. The results of the phylogenetic analysis are shown in figure 3. Human and mosquito strains clustered with Indian strains, and they contained a change (Ala226Val) in the membrane fusion glycoprotein E1 that had also been found in the Indian Ocean variant of the African genotype of CHIKV.

Discussion

This outbreak of CHIKV infection, outside a tropical country, was probably begun by a man from India, who developed a febrile syndrome 2 days after his arrival in Italy. He had high titres of antibodies against CHIKV at the time of examination (early September) and was probably highly viraemic when visiting his relatives (late June) in the village where the epidemic began. The phylogenetic analysis showed that the strain that caused this outbreak was similar to the strains detected on the Indian subcontinent[5] and that it contained the same mutation found in a variant in the Indian Ocean islands,[7,17] which is thought to be better adapted to *A albopictus* than are other variants. The hypothesis that this variant has a high virus-vector fitness seems to be confirmed by both the successful introduction and rapid spread of the infection from one infected human host and by the further occurrence of other smaller clusters in different localities in the same province yet located several kilometres from the two villages initially affected.

Samples of *A albopictus* mosquitoes from the villages were found to be positive for CHIKV sequences. The high density of the vector at the time of arrival of the index case, as anecdotally reported by villagers, was probably a major determinant of the outbreak. Actually, the population of *A albopictus* was already well established in scattered foci in Ravenna province (an average of >80% positive ovitraps), but had only recently enlarged its peripheral area to include these villages, which might explain the high vector density (ie, before control measures had been implemented). The presence of *A albopictus* in Italy is not surprising. The mosquito was first documented in Genoa (northwestern Italy) in 1990,[18] and the presence of a breeding population was first reported near Padua (northeastern Italy) in 1991.[19] The source of infestation was identified as a warehouse of a tyre retreading company that had imported used tyres infested with mosquito eggs from Georgia, USA.[20] Unfortunately, despite efforts made to control the spread of *A albopictus* mosquitoes, they rapidly colonised almost the entire country,[21,22] showing a high degree of fitness.

The peak of the outbreak occurred during the third week of August, more than 6 weeks after the onset of symptoms in the first locally acquired case, and 8–9 weeks after the onset of symptoms in the presumed