Development of a Sensitive Real-Time Reverse Transcriptase PCR Assay with an Internal Control to Detect and Quantify Chikungunya Virus


Background: The chikungunya virus (CHIKV; Alphavirus, Togaviridae) has emerged in the south Western Indian Ocean since early 2005. A major outbreak of CHIKV infection occurred in Réunion Island, where the virus is transmitted by Aedes albopictus mosquitoes. Facing an outbreak of unprecedented magnitude, we developed a rapid, sensitive, and reliable assay for the detection and quantification of CHIKV in plasma samples.

Methods: A dual-color TaqMan 1-step reverse transcriptase PCR assay was developed in a LightCycler 2.0 system. A coextracted and coamplified chimerical RNA sequence was used as an internal control (IC) to eliminate false-negative results. The CHIKV-specific and IC probes were labeled with 6-carboxyfluorescein (530 nm) and the wide span dye DYXL (705 nm), respectively, eliminating the need for color compensation. A synthetic RNA was used as an external calibrator for CHIKV absolute quantification.

Results: The detection limit was 350 copies/mL (3 copies/capillary). A further improvement to ~40 copies/mL was obtained by use of a larger volume of plasma. The assay specificity was confirmed in vitro and in silico. CHIKV in 343 patients was present at viral loads >10^8 copies/mL, mainly in newborns and seniors >60 years old. Long viremic phases of up to 12 days were seen in 6 patients.

Conclusions: The assay is rapid, CHIKV-specific, and highly sensitive, and it includes an IC. It proved useful to detect and quantify CHIKV during the Réunion Island epidemic. The assay might be applicable to other CHIKV epidemics, especially in the Indian subcontinent, where an extensive outbreak is ongoing.

Chikungunya virus (CHIKV) is an arthropod-borne virus (arbovirus) transmitted to humans by mosquitoes belonging to the Aedes genus. CHIKV is an enveloped, single-strand positive RNA Alphavirus in the Togaviridae family (1). CHIKV infection produces a human disease characterized by fever, arthralgia, and maculopapular rash (2). Other symptoms, such as headache, asthenia, nausea, vomiting, myalgia, and dysgeusia, have also been described (2, 3). Severe clinical signs such as hepatitis or encephalopathy were also reported during the Réunion 2005–2006 outbreak (2, 3). Symptoms usually appear after 2–4 days of incubation and may disappear after a few days, but arthralgia may persist for months or even years (4).

Since the virus was isolated in Tanzania in 1952 (5), many CHIKV outbreaks have occurred in Eastern, Southern, Central, and Western Africa (6, 7). CHIKV was also reported in Asia (8–10) and India (11). Phylogenetic analyses based on partial glycoprotein E1 nucleotide sequences revealed the existence of 3 distinct phylogroups: West African, East-Central-South African (ECSA), and Asian (3, 7). It was also shown that CHIKV initially originated in Africa and was introduced in Asia (7). Recent extensive outbreaks of chikungunya fever in the Indian subcontinent confirmed the reemergence of the virus.
virus in 2006 in 3 states of India (12). Since the beginning of 2005, CHIKV has emerged in several Indian Ocean islands (Comoros, Réunion, Mauritius, Seychelles, and Madagascar) (13, 14). The vector involved in Réunion Island was the Asian tiger mosquito Aedes albopictus (15). The analysis of 6 complete genome sequences and 127 E1 sequences showed that the Indian Ocean isolates share a common origin and belong to a distinct cluster within the ECSA phylogroup (3). Moreover, a specific mutation in the E1 sequence (A226V) was selected during the Indian Ocean outbreaks. The mutation was absent in the initial strains (March to June 2005) and was subsequently observed in >90% of strains after September 2005. The mutation is believed to confer to the virus a selective advantage in A. albopictus (3).

Diagnosis of CHIKV infection is mostly based on serological and PCR techniques (1). Virus cultures followed by detection of viral antigens is a sensitive method but must be performed under BSL3 biosafety conditions and is time consuming. This method is therefore used only for virus identification at the beginning of an epidemic. Serological methods are reliable but are not appropriate in early stage infection, i.e., before 5–6 days after the clinical onset. Nucleic acid amplification by PCR is an appropriate diagnostic tool at an early stage of infection, while the patient is viremic (16). Real-time reverse transcriptase PCR (RT-PCR), in particular, has been developed for the detection of several arboviruses (17–20), including CHIKV (21). Real-time RT-PCR allows the initial genome concentration to be quantified and therefore the viral load to be accurately determined. However, RT-PCR detection assays should include the extraction and the amplification of an internal control (IC) because numerous PCR inhibitors may be present in human samples (16, 22).

We developed a rapid, sensitive, and reliable RT-PCR assay that involves the coextraction and amplification of an IC. We used the assay as a diagnostic tool for the detection and quantification of CHIKV genomes in plasma samples. We determined the analytical sensitivity and specificity of the assay and the viral load for patients of different ages with typical chikungunya symptoms.

Materials and Methods

Patients and samples
All the CHIKV-positive patients reported in this study (n = 343) were admitted to the hospital between October 2005 and June 2006 with chikungunya acute symptoms. A total of 169 blood samples were obtained from the emergency care unit and 174 from other units of the hospital. Patients from the emergency unit had detailed clinical files.

CHIKV-negative samples (n = 112) were tested to evaluate the specificity of the assay. Among them, 10 were positive for, respectively, human enterovirus, HIV, hepatitis C virus, hepatitis B virus, cytomegalovirus, Epstein–Barr virus, herpes simplex virus, varicella-zoster virus, dengue virus, and West Nile virus. The 102 remaining samples were obtained during the 2005 southern hemisphere winter, when CHIKV transmission was low, from patients admitted to the hospital for other diseases than chikungunya fever.

Whole blood was collected in 5 mL EDTA-Vacutainer Tubes (Becton Dickinson Vacutainer System) and centrifuged 15 min at 2000g. The clarified plasma samples were stored at −80°C as 250-µL aliquots.

Extraction of viral RNA
Viral RNA was extracted from 200 µL of thawed samples using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) according to the manufacturer’s instructions. To improve sensitivity, large-volume extractions were also performed from 500 µL of thawed samples using the Large Volume MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics). In both cases, nucleic acids were recovered in 50 µL of elution buffer and immediately subjected to molecular analysis. For routine analysis and large-volume analysis, the lysis buffer was enriched with IC RNA (see below) at a concentration of 1.83 × 10^5 copies/mL and 3.65 × 10^4 copies/mL, respectively. The final concentration in both cases was 282 copies/capillary. The lysis buffer enriched with IC RNA was cooled to 4°C before extraction.

Single-step SYBR green RT-PCR
We performed RT-PCR amplifications with the LightCycler® 2.0 system (Roche Diagnostics). Four primers located in the glycoprotein E1 gene were selected among published sequences (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue8). Assay conditions were optimized using the LightCycler RNA Master SYBR Green I Kit (Roche Diagnostics). The 20-µL reaction mix contained 2 µL of positive control RNA, 7.5 µL of LightCycler RNA Master SYBR Green I, 3.25 mmol/L Mn^2+ , and each forward and reverse primer at a concentration of either 150, 300, or 450 nmol/L. The thermal cycling consisted of a reverse transcription at 61°C for 20 min, followed by 40 cycles at 95°C for 5 s, 57°C for 10 s, and 72°C for 15 s. The fluorescence was measured at 530 nm. After amplification, a melting curve analysis was performed to identify the amplicons by their specific melting temperatures (T_m). The melting curve analysis consisted of a denaturation step at 95°C for 5 s, a temperature decrease to 55°C for 15 s, and a temperature increase to 95°C (0.1°C/s) with continuous measurement of the fluorescence.

TaqMan® real-time RT-PCR
Amplifications were performed using the LightCycler 2.0 system (Roche Diagnostics). Assay conditions were optimized using various primer concentrations and the LightCycler RNA Master Hybridization Probe Kit (Roche Diagnostics). The 20 µL reaction mix contained 2 µL of
extracted RNA, 7.5 μL of LightCycler RNA Master Hyb-Probe, 3.25 mmol/L Mn²⁺, 450 nmol/L ChikF1 primer, 150 nmol/L ChikR1 primer, 150 nmol/L ChikProb (5’-carboxyfluorescein-3’ TAMRA), and 50 nmol/L ICProb (5’-DyXL-3’ BHQ2) fluorescent-labeled probes (TibMolBiol). The thermal cycling consisted of a reverse transcription at 61 °C for 20 min followed by 45 cycles at 95 °C for 5 s and 60 °C for 15 s. The fluorescence was measured at 530 nm.

Large-volume assays were performed with the TaqMan RNA Amplification Kit (Roche Diagnostics). The 100 μL reaction mix contained 10 μL of extracted RNA, 20 μL of TaqMan RNA Reaction Mix, 2 mmol/L Mn²⁺, 450 nmol/L ChikF1 primer, 150 nmol/L ChikR1 primer, 150 nmol/L ChikProb, and 50 nmol/L ICProb. RT-PCR cycling was carried out as follows: precycle #1, 50 °C for 150 s; precycle #2, 95 °C for 1 min; and precycle #3, 60 °C for 30 min (reverse transcription). Precycles were followed at 95 °C for 10 s and 60 °C for 15 s. The fluorescence was measured at 530 nm.

The data were analyzed with LightCycler Software Version 4.05. The Cₜ (cycle threshold) for each sample was calculated using the 2nd derivative maximum algorithm. Each test run included a water negative control and a positive control.

EXTERNAL CALIBRATOR
A synthetic RNA was used as an external calibrator for CHIKV absolute quantification. The target sequence was amplified by RT-PCR, purified using the GeneClean Turbo Kit (Qiogene), and cloned into the T7 expression vector pGEM-T Easy® (Promega). For in vitro transcription, a 524-bp DNA fragment, encompassing the complete CHIKV target sequence and the T7 promoter, was amplified with vector-specific primers. The resulting fragments were purified using the GeneClean Turbo Kit (Qiogene) and in vitro transcribed using the Riboprobe® T7 System (Promega). The 439-bp RNA transcripts were purified using the RNeasy® MinElute™ Kit (Qiagen) and the amount of RNA was estimated using the Quant-iT™ RiboGreen® RNA Kit (Molecular Probes) as described by the manufacturers.

IC
A chimerical RNA was used as an IC. It consisted of a 45-bp enterovirus target sequence (Coxsackievirus A16, position 537 to 581, GenBank accession no. U05876), lengthened by a 5-step PCR using 5 pairs of partially overlapping oligonucleotides. The resulting 209-bp DNA fragment was flanked by ChikF and ChikR primer target sequences and displayed the same GC content as the corresponding CHIKV DNA fragment. The IC DNA was cloned, in vitro transcribed, and treated with DNase to remove all residual DNA contamination. The RNA amount was estimated as described above, and the transcript was stored at −80 °C in PCR-grade water.

The IC RNA sequence was RT-PCR amplified using the ChikF1 and ChikR1 primers and detected by the IC specific fluorescent-labeled probe ICProb (5’ DYXL-3’ BHQ2; TibMolBiol). DYXL is excited at 470 nm and detected at 705 nm. BHQ2 is a nonfluorescent “black hole” quencher.

SEQUENCE ALIGNMENTS
Multiple sequence alignments of E1 gene sequences were performed using ClustalX 1.8 (23) to evaluate the specificity of the CHIKV primers and probe (see Table 1 in the online Data Supplement). A total of 47 sequences were retrieved from the GenBank database, and 127 partial E1 nucleotide sequences were available from a previous study (3).

RESULTS

DETAILED DESIGN AND SCREENING OF PRIMER PAIRS USING A SINGLE-STEP SYBR GREEN RT-PCR
Primer combinations were tested using a single-step SYBR Green RT-PCR. The concentration for each primer and the thermal cycling were optimized to increase sensitivity. The specificity was verified by melting curve analysis of the amplicons. The Tₘ values for primer dimers were <75 °C, and the Tₘ for the CHIKV-specific fragment was 82 °C. The optimal primer concentrations were 450 nmol/L for the forward primer and 150 nmol/L for the reverse primer. The best sensitivity (smallest Cₜ value) was achieved with the ChikF1/ChikR1 pair. This result was in accordance with the E1 sequences, which were determined from 127 patients during the Indian Ocean outbreak (3). No mismatch was observed between the ChikF1/ChikR1 primers and the Indian Ocean CHIKV sequences. The Cₜ value was 1 cycle higher when we used any of the other primer combinations instead of ChikF1/ChikR1.

To develop a TaqMan RT-PCR assay, the specificity of the TaqMan probe (ChikProb) was also checked using sequence alignments. No mismatch was observed with the Indian Ocean CHIKV sequences. However, 1 Indian strain (GenBank accession no. DQ888620) and 4 West African strains (AF192891, AF192892, AF192893, and AY726732) showed 1 and 2 mismatches, respectively (see Table 1 in the online Data Supplement).

SENSITIVITY AND REPRODUCIBILITY OF THE TaqMan RT-PCR ASSAY
Ten-fold serial dilutions of the RNA external calibrator were done in CHIKV-negative plasma, resulting in concentrations ranging from 10⁶ to 10⁸ copies/mL. Dilutions of RNA were extracted and amplified in triplicates in the same run, to evaluate the intraexperimental reproducibil-
ity. The mean C<sub>T</sub> values were plotted against the copy number to establish a calibration curve (see Fig. 1 in the online Data Supplement). The correlation coefficient was >0.998 and the slope was −3.38. The amplification efficiency, calculated as 10<sup>−1/slope</sup>, was 1.97. The assay was reproducible; SDs of C<sub>T</sub> values were <0.31 (Table 1). The detection limit was between 10<sup>2</sup> and 10<sup>3</sup> copies/mL. The linear detection range was 10<sup>3</sup> to 10<sup>9</sup> copies/mL. To refine the estimate of the detection limit, additional dilutions of the RNA calibrator were extracted and amplified in triplicate. The last dilution to be amplified with a detection rate of 100% corresponded to a mean (SD) of 353 (17) copies/mL, according to the calibration curve (see Fig. 1 in the online Data Supplement), corresponding to 2.9 (0.14) copies/capillary. This finding is consistent with the Poisson distribution, which predicts that 95% of replicates will be positive for a mean concentration of 2.9 copies per reaction mix (24). When using the large-volume protocol, the detection threshold was decreased to 42 (4.2) copies/mL, which corresponded to 4.21 (0.4) copies/capillary. The interassay variability was evaluated by amplifying a sample containing 10<sup>5</sup> copies/mL in 15 different runs. The mean C<sub>T</sub> value was 29.01, with a low SD of 0.26 cycles (data not shown).

Implementation of the IC

A chimerical RNA was used as IC to eliminate false-negative results due to PCR inhibition and extraction failure. The RT-PCR protocol consisted of a dual-color assay with heterologous probes to detect the CHIKV target and the IC sequences, which were both amplified by ChikF1/ChikR1. Several IC concentrations were tested to decrease competition with CHIKV RNA amplification. The optimal IC concentrations that allowed the same sensitivity for CHIKV target amplification were 1.83 × 10<sup>5</sup> copies/mL and 3.65 × 10<sup>4</sup> copies/mL for the routine and large-volume protocols, respectively. This corresponded, after extraction, to a final concentration of 282 copies/capillary in both cases. The optimal concentrations were obtained using the lowest IC probe concentration that allowed reliable detection (50 nmol/L). CHIKV target and IC sequences were specifically detected at 530 nm and 705 nm, respectively, with no fluorescence cross-talk. Nevertheless, inhibition of the IC amplification occurred in the presence of high CHIKV RNA target concentrations. This was not detrimental, however, because IC is used to monitor false-negative results.

CHIKV TaqMan RT-PCR specificity

In addition to the in silico specificity described above, a panel of arboviruses and other viruses commonly found in blood was used to verify the in vitro specificity of the CHIKV TaqMan RT-PCR after 45 amplification cycles. No amplification was obtained from samples that were positive for human enterovirus, HIV, hepatitis C virus, hepatitis B virus, cytomegalovirus, Epstein–Barr virus, herpes simplex virus, varicella-zoster virus, dengue virus, and West Nile virus. Moreover, 102 CHIKV-negative samples obtained from patients admitted to the hospital for diseases other than chikungunya fever tested negative using the CHIKV TaqMan RT-PCR assay. In each case, the IC was successfully extracted, amplified, and detected.

CHIKV quantification from infected patients

We used the CHIKV TaqMan RT-PCR assay to test 343 patients with acute symptoms of chikungunya fever; all were positive. The proportional distribution of age classes was plotted against viral load (Fig. 1). The proportion of patients <20 years of age increased with viral load, especially for newborns (white circles). Inversely, the proportion of patients between 20 and 60 years of age decreased with viral load. Patients with viral loads >10<sup>8</sup> copies/mL were mainly newborns and adults >60 years olds (40.80% and 40.81%, respectively).

The 343 patients included 169 emergency care unit patients, from whom samples were collected between the

<table>
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<th>RNA synthetic standard concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;, mean (SD)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Detection rate</th>
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<tr>
<td>1.14 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>10.22 (0.23)</td>
<td>100</td>
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<tr>
<td>1.14 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>13.38 (0.26)</td>
<td>100</td>
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<tr>
<td>1.14 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>16.74 (0.18)</td>
<td>100</td>
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<tr>
<td>1.14 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>20.48 (0.11)</td>
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<td>1.14 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>100</td>
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<td>100</td>
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<td>1.14 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>32.3</td>
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<sup>a</sup> Ten-fold serial dilutions of the RNA external calibrator were done in CHIKV CHIKV-negative plasma (copies/mL), submitted to RNA extraction, and tested in triplicates in the same routine run to evaluate the intra-experimental reproducibility.

<sup>b</sup> SD was calculated using the C<sub>T</sub> values.

![Fig. 1. Proportional distribution of age classes against CHIKV load for 343 studied patients.](image)

*White circles represent babies less than 1 year of age. The age class percentages (%) are given inside proportional circles. The population sizes (n) are indicated.*
1st and 12th day after the onset of the symptoms. Viral load ranged from \(2.91 \times 10^4\) to \(8.55 \times 10^8\) copies/mL. For 89% of these patients blood was collected within the first 3 days after the symptoms appeared (Fig. 2). These emergency care unit patients were mainly adults (98.7% >16 years old), and mean age increased with viral load in a nonlinear fashion (Fig. 3).

**Discussion**

Since early 2005, Réunion Island has been suffering from a large-scale outbreak of chikungunya fever. CHIKV has infected (one-third) of the population, i.e., \(~300\) 000 people. Several patients developed severe clinical signs such as meningoencephalopathy (3), and 249 death certificates mentioned “chikungunya” as a comorbidity factor (25). Mother-to-child transmission of the virus was reported (26). This outbreak of unprecedented magnitude necessitated the development of a rapid, sensitive, and reliable CHIKV-detection assay.

Our dual-color TaqMan RT-PCR technique was CHIKV specific, as verified both in silico and on 112 clinical samples. We achieved better sensitivity and wider linear range than previously reported (21, 27). In addition, the use of a coextracted and coamplified IC allowed the detection of false-negative results due to PCR and/or extraction failures. To eliminate the need for color compensation in the LightCycler 2.0 platform, we chose DYXL as an IC fluorescent reporter. Its emission spectrum (705 nm) does not overlap with the 6-carboxyfluorescein emission spectrum (530 nm). Compared with conventional RT-PCR techniques, our assay was rapid and could be performed in 2 h, including the MagNA Pure nucleic acid extraction. Furthermore, the use of single closed capillaries eliminated the risk of contamination.

No RT-PCR inhibition was observed for the 343 plasma samples tested during this study. However, inhibition occurred for 2% of CHIKV-positive and -negative plasma samples tested in routine analysis since the beginning of the outbreak. This result emphasizes the importance of using an IC to provide a reliable clinical diagnosis. For instance, the use of a coextracted IC enabled the detection method to be adapted to a maternal milk specimen, as 100% were inhibitory using the plasma extraction technique (data not shown).

Our routine assay allowed the detection of 353 (17) copies/mL in plasma samples [2.9 (0.14) copies/capillary] and was therefore sensitive enough to diagnose all clinical cases, because we found no viral load <10^4 copies/mL in all 343 plasma samples. The large-volume protocol reached an even greater sensitivity of 42 (4.2) copies/mL. This increased sensitivity was useful for types of samples other than plasma, such as cerebrospinal fluids, in which viral loads were, on average, 25 000 times lower than in blood (28). The large-volume protocol was also useful for blood donor screening. Blood donation had stopped in Réunion Island, because no reliable test was available before we developed our assay. The high sensitivity was
important for detection of viremic donors without clinical signs (incubation period or asymptomatic cases). These individuals might have low viral loads, as previously shown in asymptomatic patients infected with the West Nile virus (29, 30).

In our study, 6 patients showed a longer CHIKV viremic phase than previously reported (5–6 days) (31). CHIKV RNA was detected up to 12 days after the clinical onset, with high viral load (6 $10^5$ to 1.3 $10^6$ copies/mL). Longer viremia might be due to a delayed or less efficient immune response, because CHIKV-specific IgM were not detected in these patients (data not shown). No correlation with age was observed; patients were of various ages: 32, 46, 52, 62, 84, and 86 years old. The high viral load at 12 days suggested that the virus might circulate for an even longer period.

In our study, patients with high viral loads (>10^6 copies/mL) were mainly newborns (40.8%) and older adults >60 years old (40.81%). These 2 populations may have a less efficient immune response, allowing a higher rate of virus replication.

Sequence alignments showed that our RT-PCR assay is applicable to the entire Indian Ocean region and the Indian Subcontinent. Isolates from the Southern Indian Ocean outbreak and the current outbreak in India are closely related and belong to the African genotype (3, 11, 12, 32).

Our TaqMan RT-PCR assay can also be useful to detect CHIKV among travelers coming from epidemic regions where the ECSA genotype circulates. Transmission in Réunion Island began with travelers coming from the Comoros islands. During 2006, numerous imported cases of chikungunya fever were diagnosed among travelers returning from Réunion, Seychelles, Mauritius, Madagascar, and India (31, 33). A total of 766 imported cases of chikungunya have been identified in mainland France (i.e., excluding overseas departments and territories) since the beginning of the Indian Ocean outbreak (34). Because A. albopictus has already been introduced into several European countries (35–37), autochthonous CHIKV transmission is theoretically possible but has not been reported to date (33). The risk for autochthonous CHIKV transmission by mosquitoes has been taken into consideration in mainland France, and reinforced surveillance has been organized in the areas where the mosquito is present.

In conclusion, our laboratory developed a highly sensitive and reliable dual-color TaqMan 1-step RT-PCR assay for the detection and the quantification of CHIKV in plasma samples. In several tropical regions, arbovirus diseases are emerging or reemerging in a cyclical way (11, 15, 32, 38). The ongoing chikungunya epidemic in the Indian Ocean, as well as the 1978 (39) and 2004 Dengue virus-1 (40) outbreaks in Réunion Island provided evidence that other arbovirus outbreaks are likely to occur. Based on such perspective we are currently developing a multiplex real-time RT-PCR assay for the detection of a range of arboviruses.

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References


