A Chikungunya virus (CHIKV) outbreak continues in India. Monitoring of the clinical features of CHIKV infection is an important component of assessing the disease process. Diagnosis is usually made by an immunoglobulin M (IgM)/IgG enzyme-linked immunosorbent assay (ELISA). However, these assays have extremely low sensitivities for the detection of infection in the majority of CHIKV patients during the acute stage of infection (during the 1 to 4 days after infection). In our laboratory, a sensitive ELISA protocol for antigen detection has been developed for the detection of CHIKV infection in the acute stage, and in the present study we assessed the usefulness of this ELISA-based system for the detection of CHIKV infection. We performed a prospective, double-blinded study of 205 Indian patients with suspected CHIKV infection in the Nagpur District. All patients underwent a full clinical assessment, and their serum samples were analyzed for the presence of antigens and of IgM and IgG by an ELISA protocol. In patients with CHIKV infection, the sensitivity of antigen detection was 85%, which was significantly higher (P < 0.001) than that of IgM (17%) or IgG (45%) detection. The sensitivity of IgM (20%) or IgG (25%) detection was significantly lower than that of the antigen assay (95%) for patients with acute infections (i.e., from day 1 to day 5 after infection). Antigen detection not only gives a positive confirmatory result in the early phase of the disease, but it is also useful in the prodromal and subclinical stage and may be useful for field applications for the rapid detection of CHIKV infection.
the assessment of patients with suspected CHIKV infection (8). However, the sensitivity of the IgM-capture ELISA is low for the majority of patients in the acute stage of illness (days 1 to 5); therefore, a negative IgM-capture ELISA result does not rule out a diagnosis of CHIKV infection. The specificity of the IgM-capture ELISA is also limited because of cross-reactivity with other alphavirus-related infections. IgG cannot be detected in CHIKV-infected patients in the acute stage.

A rapid antigen detection test that uses ELISA for the detection of CHIKV infection may be a more accurate diagnostic method for patients in the acute stage of infection. In the present study, we compared the usefulness of antigen detection by ELISA with that of antibody (IgM and IgG) detection by ELISA. Our goal was to determine whether antigen detection by ELISA improves the diagnostic assessment of patients with CHIKV infection in routine medical practice. We performed antigen and IgM and IgG detection at the time of initial assessment and compared the test results with the final clinical, immunological, and molecular (real-time reverse transcription-PCR [RT-PCR]) diagnoses.

This study was done in Nagpur, India, the location of the CHIKV epidemic, and the Institutional Ethics Committee of the Central India Institute of Medical Sciences (CIIMS), Nagpur, India, approved the study.

MATERIALS AND METHODS

Selection of patients. Patients from the village of Bhilgaon (Nagpur District) were enrolled from July 2006 through September 2006 at CIIMS. All patients between the ages of 5 and 85 years with symptoms considered to be the clinical features of CHIKV infection were included in this study; these symptoms included fever, headache, myalgia, joint pain with or without swelling, and the presence or absence of rash on the body. An acute case of CHIKV infection was defined as any case with clinical features consistent with Chikungunya fever and in whom CHIKV infection was confirmed either by RT-PCR or by real-time PCR or virus isolation. Serum samples from 205 participants, comprising 55 individuals from the village of Bhilgaon (a rural locality) and the remaining 150 individuals from the CIIMS in Nagpur (an urban locality), were studied. A slight majority of the subjects (122; 59%) were male. The median age was 45 years (range, 5 to 85 years). All patients gave oral consent. Written consent was deemed unnecessary since all diagnostic tests are routinely used in clinical practice. Clinically, the patients were divided into the categories of those with confirmed CHIKV infection, those with suspected CHIKV infection, and those without CHIKV infection (non-CHIKV infection), as outlined in Table 1.

Preparation of anti-CHIKV. Pooled sera from patients infected with CHIKV were collected, and IgG was purified by protein G affinity column chromatography (IgG purification kit; Bangalore Genei, India), according to the manufacturer’s instructions, and was used at a dilution of 1:10,000. The specificity of the antibody was evaluated by ELISA by using the antigens of different alphaviruses (CHIKV and Ross River virus [RRV]).

Antigens. The CHIKV and RRV antigens used in the present study were a kind gift from P. V. L. Rao, Department of Virology, Defense Research and Development Establishment (DRDE; Gwalior, India).

An Indian strain of CHIKV, strain ISW HYD06 (GenBank accession number 876190), was used to prepare the antigens. The virus belonging to the novel East-Central South African (ECSA) genotype was isolated from a patient from Hyderabad, India, during the 2006 epidemic, and its identity was confirmed by RT-PCR and serology. CHIKV ISW HYD06 was initially isolated in C6/36 cells (a cell line derived from the larvae of Aedes albopictus) and was then serially passaged in Vero cells to increase its adaptability as well as the virus titer. The virus-infected culture supernatant was clarified by centrifugation at 3,000 rpm for 10 min. The clarified infected culture supernatant was then concentrated by polyethylene glycol (PEG) precipitation. The purification of the PEG-pelleted viral preparation was carried out by discontinuous sucrose gradient centrifugation in an ultracentrifuge with 50% and 20% sucrose cushions by the method of Gould and Clegg (2a). The sucrose gradient-purified Chikungunya virus antigen used in this study was obtained from DRDE.

Standardization of antigens. Antigen titration was carried out by ELISA with the human anti-CHIKV IgG in order to prepare the virus antigens (Fig. 1). Briefly, the wells of the ELISA plate (Nunc) were coated with 100 μl of viral antigen (dilution 1:2) in phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20 (PBS-T) for 90 min and then were blocked with 0.5% bovine serum albumin (BSA) in PBS-T for 60 min. After 60 min of incubation at room temperature, the wells were washed with PBS-T and were then reacted with anti-CHIKV IgG (pooled from sera from CHIKV-infected patients), and the plate was incubated at 37°C for 60 min. The wells were washed, the secondary antibody (goat anti-human IgG–horseradish peroxidase conjugate; dilution, 1:10,000) was added, and the plate was incubated for 60 min at 37°C. After another washing with PBS-T, 100 μl of a tetramethylbenzidine (TMB)-H2O2 substrate solution was added to the wells, which were then incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 μl of 2.5 N H2SO4. The absorbance of each well was read at 450 nm.

CHIKV antigen detection. One hundred microliters of serum samples (dilution, 1:100) from CHIKV-infected patients was added to the microtiter wells, and then the wells were blocked with 0.5% BSA in PBS-T for 60 min. After the samples were washed with PBS-T, anti-CHIKV IgG was added (dilution, 1:10,000) and the plates were incubated at 37°C for 60 min. After incubation, the wells were washed and goat anti-human IgG–horseradish peroxidase secondary antibody (dilution, 1:10,000) was added. The samples were then incubated for 60 min at 37°C. After another wash with PBS, 100 μl of the TMB-H2O2 substrate solution was added to the wells, which were incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 μl of 2.5 N H2SO4. The absorbance of each well was read at 450 nm.

CHIKV IgM and IgG detection. The IgM and IgG antibodies to CHIKV were detected by ELISA. An indirect ELISA protocol allowed the detection of IgG and IgM using a purchased ELISA kit (BDI, Netherlands) with a procedure similar to that of the IgG-positive samples. The antigen was diluted 1:2 in 0.05% Tween 20 in PBS. The IgG or IgM capture ELISA was performed in 96-well microtiter plates (Maxisorp; Nunc, Denmark). The plates were coated with 100 μl of the diluted antigen for 16 h at 4°C and were then washed two times with PBS-T. The plates were blocked with 100 μl of 0.5% BSA in PBS-T for 1 h at 37°C. After blocking, the plates were washed two times with PBS-T and incubated with 100 μl of serum diluted 1:10,000 for 1 h at 37°C. The plates were then incubated at room temperature for 30 min after washing two times with PBS-T. The plates were incubated for 30 min at room temperature in 100 μl of goat anti-human IgG–horseradish peroxidase conjugate (dilution, 1:4,000). The plates were washed, and 100 μl of tetramethylbenzidine (TMB) substrate solution was added to the wells, and the reaction was stopped after 5 min with 100 μl of 2.5 N H2SO4. The absorbance of each well was read at 450 nm.

FIG. 1. Recruitment and diagnostic classification of participants.

| Symptom | CHIKV Infection | Non-CHIKV Infection |
|---------|-----------------|---------------------|
| Fever   | 30 patients     | 0                   |
| Cough/cold | 20 patients | 0                   |
| Upper respiratory infection | 10 patients | 0                   |
| Generalized body ache | 0 patients | 0                   |
| Headache | 10 patients     | 0                   |
| Extreme fatigue | 0 patients | 0                   |
| Rash with pruritus | 0 patients | 0                   |
| Myalgia  | 10 patients     | 0                   |
| High-grade fever | 0 patients | 0                   |
| Joint pain with and without swelling | 0 patients | 0                   |
| Cough/cold | 0 patients | 0                   |
| Upper respiratory infection | 0 patients | 0                   |
| Rash with pruritus | 0 patients | 0                   |
| Headache | 0 patients     | 0                   |
| Maculopapular rash | 0 patients | 0                   |

TABLE 1. Diagnostic classification of patients presenting with Chikungunya fever

| Confirmed Chikungunya fever | Suspected Chikungunya fever | No Chikungunya fever |
|-----------------------------|-----------------------------|----------------------|
| RT-PCR and/or real-time PCR positive or virus isolation | Low-grade fever | Fever |
| High-grade fever | Joint pain with and without swelling | Cough/cold |
| Headache | Extreme fatigue | Upper respiratory infection |
| Joint pain with and without swelling | Rash with pruritus | Generalized body ache |
| Myalgia | Headache | |
| Maculopapular rash | Nausea/vomiting | |

- Data from: KASHYAP ET AL. CLIN. VACCINE IMMUNOL.
and IgM antibodies directed against CHIKV antigens. The viral antigens (dilution, 1:2) in PBS-T were coated onto the microtiter wells. After 90 min of incubation at room temperature, the wells were washed with PBS-T, and the plates were incubated for 60 min. After incubation of the diluted patient serum, 100 μl of goat anti-human IgG or IgM conjugated with horseradish peroxidase was added. After another washing with PBS, 100 μl of TMB-H2O2 substrate solution was added to the wells, which were incubated at room temperature for approximately 10 min. The reaction was then stopped with 100 μl of 2.5 N H2SO4. The absorbance of each well was read at 450 nm.

**Real-time RT-PCR.** Real-time RT-PCR of samples was performed at DRDE.

**Primers.** The oligonucleotide primers used for real-time amplification of CHIKV were designed from the E1 gene of African CHIKV prototype strain S-27 (GenBank accession number AF369024). The potential target regions were selected by identifying highly conserved regions among strains belonging to the different CHIKV genotypes by using the ClustalW program available in the Lasergene 5 package (DNAStar). In addition, comparative analysis with other related alphaviruses, namely, O’Nyong Nyong virus (ONNV), Semliki Forest virus (SINV), Mayaro virus (MAYV), and RRV was also attempted, and maximum care was taken to reduce the cross-reactivity due to sequence similarities.

**RNA extraction.** The genomic viral RNA was extracted from 140 μl of supernatant of a culture infected with a known number of PFU of virus and 140 μl of serum from each patient by using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany), according to the manufacturer’s protocols. The viral RNA was eluted from the QIAamp columns in a volume of 70 μl of elution buffer and was stored at −70°C until it was used.

**SYBR green I-based real-time RT-PCR.** A SYBR green I-based one-step real-time quantitative RT-PCR amplification was performed in an MX 3000P quantitative PCR system (Stratagene). Following optimization with standard RNA by using a brilliant SYBR green single-step quantitative RT-PCR master mix (Stratagene), the test samples were assayed in 25-μl reaction mixtures containing 12.5 μl of 2× reaction mixture, 0.4 μl of reference dye (carboxy-X-rhodamine), 1 μl (10 pmol) of each forward and reverse primer, 0.1 μl of RNA, 0.1 μl of reverse transcriptase, and 9.0 μl of nuclease-free water. No-template, no-primer, and buffer controls were also included. The thermal profile consisted of 30 min of reverse transcription at 50°C for 1 cycle and 10 min of polymerase activation at 95°C, followed by 40 cycles of PCR at 95°C for 30 s, 55°C for 60 s, and 72°C for 30 s. Following the amplification, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting temperature (Tm) with the melting curve analysis software of the MX3000 program, according to the instructions of the manufacturer.

**Conventional RT-PCR.** In order to determine the sensitivity of the real-time RT-PCR assay, a conventional RT-PCR was performed with the same primer sets targeting the 205 bp of the E1 gene of the viral genome. The amplification was carried out in a 50-μl total reaction volume by using an Access Quick RT-PCR kit (Promega), according to the manufacturer’s protocol.

**Statistical analysis.** The results are expressed as the means ± standard deviations (SDs), together with the range. Comparisons of the proportions of positive results were made between the different tests and subgroups by using the Kruskal-Wallis test (nonparametric analysis of variance) with the Dunnett post-test. A P value of less than 0.05 was considered significant. Cutoff values for the absorbance of the CHIKV antigen, IgM, and IgG were calculated by using the mean + SD of the absorbance of the CHIKV antigen, IgM, and IgG for the healthy control group. The sensitivity (true-positive rate) for the test was calculated as [the number of samples in the CHIKV-infected group with an absorbance of greater than or equal to the (mean + SD) of the absorbance for the healthy group divided by the total number of samples for the CHIKV-infected group] × 100. The specificity (true-negative rate) for the test was calculated as [the number of samples in the CHIKV-infected group with an absorbance less than the (mean + SD) of the absorbance for the healthy group divided by the total number of samples for the healthy group] × 100.

**RESULTS**

Figure 1 shows the breakdown of the number of patients in each diagnostic category. Of the total of 205 patients, 29 patients were not included in the analysis because not all the immunological tests could be performed for those individuals. Thus, 176 patients were considered for further analysis. Of these, 109 patients were in the category of individuals with confirmed CHIKV infection, 48 were in the category of indi-

**TABLE 2.** Distribution of age in the groups of patients with confirmed CHIKV infection, suspected CHIKV infection, and non-CHIKV infection

| Age (yr) | Confirmed CHIKV infection (n = 119) | Possible CHIKV infection (n = 67) | Non-CHIKV infection (n = 19) | Total (n = 205) |
|----------|----------------------------------|----------------------------------|----------------------------|---------------|
| <10      | 0 (0.8)                          | 1 (1.5)                          | 3 (16)                    | 5 (2)         |
| 10–20    | 7 (6)                            | 4 (3)                            | 3 (16)                    | 14 (7)        |
| 20–30    | 14 (12)                          | 10 (15)                          | 4 (21)                    | 28 (14)       |
| 30–40    | 28 (24)                          | 15 (22)                          | 11 (2)                    | 45 (22)       |
| 40–50    | 37 (31)                          | 22 (33)                          | 3 (16)                    | 62 (30)       |
| 50–60    | 20 (17)                          | 7 (10)                           | 21 (21)                   | 38 (18)       |
| >60      | 12 (10)                          | 8 (12)                           | 20 (10)                   | 40 (19)       |

**TABLE 3.** Assessment of clinical symptoms in the groups of patients with confirmed CHIKV infection, possible CHIKV infection, and non-CHIKV infection

| Symptom               | Confirmed CHIKV infection (n = 119) | Possible CHIKV infection (n = 67) | Non-CHIKV infection (n = 19) | Total (n = 205) |
|-----------------------|----------------------------------|----------------------------------|----------------------------|---------------|
| Fever                 | 114 (96)                         | 62 (96)                          | 15 (79)                    | 191 (93)      |
| Arthralgia without swelling | 112 (94)                     | 54 (81)                          | 8 (42)                     | 174 (85)      |
| Arthralgia with swelling       | 94 (79)                         | 55 (81)                          | 8 (42)                     | 157 (77)      |
| Myalgia                | 84 (71)                          | 38 (57)                          | 12 (63)                    | 134 (65)      |
| Rash                  | 116 (97)                         | 29 (43)                          | 145 (71)                   |
| Low back pain          | 25 (21)                          | 40 (60)                          | 9 (47)                     | 72 (35)       |
| Other                 | 08 (7)                           | 6 (9)                            | 23 (11)                    |               |

**FIG. 2.** The absorbance at 450 nm is shown with increasing concentrations of CHIKV antigen (5, 10, 20, 50, 100 ng/ml) during the standardization of the indirect ELISA method.
Healthy controls (n = 11022), CHIKV-infected group was 11% (02/19). The CHIKV antigen was detected in only one person in the healthy control group. No. (%) of subjects Absorbance

| Duration of disease | Antigen assay | IgM assay | IgG assay |
|---------------------|---------------|-----------|-----------|
| ≤5 days (n = 61)*   | 58 (95)       | 12 (20)   | 15 (25)   |
| 5–10 days (n = 38)  | 34 (89)       | 9 (32)    | 12 (32)   |
| 10–20 days (n = 38) | 27 (71)       | 3 (8)     | 30 (79)   |
| >20 days (n = 20)   | 14 (70)       | 3 (15)    | 8 (40)    |

* The values in parentheses are the number of cases of Chikungunya fever.

No. (%) of subjects

Table 5 shows the occurrence of the CHIKV antigen in sera from subjects with and without CHIKV infection by ELISA along with mean absorbance.

| Subject                          | No. (%) of subjects | Absorbance |
|----------------------------------|---------------------|------------|
| CHIKV infection (n = 157)        | 133 (85)            | 1.28 ± 0.39| 0.88–2.23 |
| CHIKV infection (n = 109)        | 98 (90)             | 1.69 ± 0.46| 0.79–2.23 |
| Possible CHIKV infection (n = 48) | 35 (73)             | 1.19 ± 0.29| 0.42–1.86 |
| Non-CHIKV infection (n = 19)     | 2 (11)              | 0.72 ± 0.19| 0.10–1.02 |
| Dengue fever (n = 05)            | 1 (20)              | 0.46 ± 0.12| 0.03–0.98 |
| Rheumatoid arthritis (n = 04)    |                     | 0.29 ± 0.10| 0.03–0.8  |
| Healthy controls (n = 10)        | 1 (10)              | 0.19 ± 0.13| 0.01–1.1  |

a The cutoff for a positive result was 0.92.
Table 6. Demonstration of IgM in sera from subjects with and without CHIKV infection by ELISA along with mean absorbance

| Subject                        | No. (%) of subjects | Absorbance     |
|--------------------------------|---------------------|----------------|
|                                | Positive for IgM<sup>a</sup> | Negative for IgM | Mean ± 2 SDs | Range     |
| CHIKV infection (n = 157)      | 27 (85)             | 130 (15)       | 0.838 ± 0.27 | 0.38–1.28 |
| CHIKV infection (n = 109)      | 19 (17)             | 92 (83)        | 0.898 ± 0.18 | 0.30–1.28 |
| Possible CHIKV infection (n = 48)| 08 (17)            | 40 (83)        | 0.692 ± 0.21 | 0.08–0.98 |
| Non-CHIKV infection (n = 19)   | 1 (05)              | 18 (95)        | 0.48 ± 0.16  | 0.08–0.68 |
| Dengue fever (n = 05)          | 1 (20)              | 4 (80)         | 0.39 ± 0.9   | 0.23–0.68 |
| Rheumatoid arthritis (n = 04)  | 1 (20)              | 4 (80)         | 0.18 ± 0.6   | 0.03–0.43 |
| Healthy controls (n = 10)      | 1 (10)              | 9 (90)         | 0.21 ± 0.13  | 0.12–0.51 |

<sup>a</sup> The cutoff for a positive result was 0.523.

Table 7. Demonstration of IgG antigen in sera from subjects with and without CHIKV infection by ELISA along with mean absorbance

| Subject                          | No. (%) of subjects | Absorbance     |
|----------------------------------|---------------------|----------------|
|                                  | Positive for IgG<sup>a</sup> | Negative for IgG | Mean ± 2 SDs | Range     |
| CHIKV infection (n = 157)        | 71 (45)             | 86 (55)        | 1.46 ± 0.28  | 0.91–2.38 |
| CHIKV infection (n = 109)        | 49 (45)             | 60 (55)        | 1.71 ± 0.32  | 0.82–2.38 |
| Possible CHIKV infection (n = 48)| 22 (46)             | 26 (54)        | 1.33 ± 0.11  | 0.39–1.83 |
| Non-CHIKV infection (n = 19)     | 9 (47)              | 10 (53)        | 0.61 ± 0.14  | 0.15–1.37 |
| Dengue fever (n = 05)            | 1 (20)              | 4 (80)         | 0.33 ± 0.12  | 0.18–1.21 |
| Rheumatoid arthritis (n = 04)    | 2 (50)              | 2 (50)         | 0.29 ± 0.11  | 0.03–0.43 |
| Healthy controls (n = 10)        | 1 (10)              | 9 (90)         | 0.11 ± 0.09  | 0.12–0.51 |

<sup>a</sup> The cutoff for a positive result was 0.95.
We assessed the clinical usefulness of the antigen, IgM, and IgG detection assays on the basis of the ELISA method for the detection of CHIKV infection in suspected CHIKV-infected patients. The study population had a high prevalence of CHIKV infection. The sensitivity of the antigen detection assay was significantly higher than that of the IgM and IgG detection assays for CHIKV-infected patients. Our results indicate that antigen detection has a higher sensitivity and specificity than IgM and IgG detection. The sensitivity of IgM detection in CHIKV-infected patients was only 17%, and therefore, IgM detection was less useful for diagnosis. Similarly, the diagnostic sensitivity of IgG detection was not very impressive (45%), whereas the sensitivity of antigen detection was 85%. The increased sensitivity of the antigen detection assay results from the fact that among CHIKV-infected patients for whom the results of all three tests are available, 126 were IgM negative but antigen positive and 68 were IgG negative but antigen positive, whereas 15 were IgG positive but antigen negative. This difference in the results may reflect differences in the infection and the time of sample collection during the infection.

The observation that the rate of positive antigen detection was highest (95%) in the acute phase (first 5 days) and then gradually diminished is an important finding, because it indicates the possibility that a definitive diagnosis of this viral disease may be obtained at a very early stage. It may also be possible to detect the disease in the prodromal or subclinical stage, when viremia is present. The detection of viral antigen throughout the course of the infection suggests the presence of a high number viral particles (viral load) in the blood.

No serum specimens from children younger than 5 years old suspected of having CHIKV infection were received. According to local physicians, they have not seen many cases of children with the symptoms of CHIKV infection. The reasons for this phenomenon are unclear.

CHIKV infections are thought to occur more commonly among elderly patients with underlying medical conditions. However, in our study, we included subjects from all age groups, including very young and very old individuals, and our results do not show such an observation or occurrence. Moreover, our tests performed similarly regardless of the patient’s age. Nevertheless, CHIKV infection has a somewhat different clinical picture in younger patients (10 to 40 years). Arthralgia and arthritis occur but are less prominent and shorter lasting, and rashes may occur in small proportions of patients.

In our study, we selected study subjects from both from rural and urban areas. It was expected that CHIKV infection would be more common in remote populations (rural areas); however, the results from our study showed a similar prevalence of CHIKV infection between the two areas. However, the biggest problem with this study is that we were unable to quantify to what extent the residents of rural areas had contact with the urban population. Similarly, age was not significantly associated with CHIKV infection. However, 50% of the study population with suspected CHIKV infection fell within the age range of 30 to 50 years, suggesting that the rate of Chikungunya fever is high predominantly in this age group. When the seroprevalence data for CHIKV infection were stratified by sex, a significant difference in prevalence was observed be-
between women and men, suggesting that the prevalence of CHIKV infection is higher in women.

The results indicate that the indirect ELISA method used in this study is sensitive, specific, rapid, and cost-effective; and it can be adopted by any laboratory with an ELISA reader and an incubator. It may thus be useful in laboratories with limited resources, especially in underdeveloped and developing countries.

Overall, the findings of our study suggest that the diagnostic sensitivity of CHIKV antigen detection by use of an ELISA-based system is higher than that of IgM and IgG detection and the ELISA-based system is helpful for the detection of antigen throughout the infection, even in the earlier stages of infection. Therefore, we conclude that CHIKV antigen detection can be very effective for diagnosis not only in the early stage of the disease but possibly also in the prodromal or subclinical stage.

ACKNOWLEDGMENTS

This work was funded by the Department of Biotechnology, Government of India, New Delhi, India, and the Central India Institute of Medical Sciences, Nagpur, India.

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