

Real-Time-PCR Assay for Diagnosis of *Entamoeba histolytica* Infection

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We developed a real-time-PCR assay utilizing a molecular-beacon probe for the detection of *Entamoeba histolytica* and compared its sensitivity to stool antigen detection and traditional PCR. A total of 205 stool and liver abscess pus specimens from patients and controls were used for this purpose, 101 (49%) of which were positive by the TechLab *E. histolytica*-specific antigen detection test, while the other 104 (51%) stool and liver abscess pus specimens were negative by the antigen detection test. DNA was extracted from the stool and liver abscess pus specimens by the QIAGEN method and the small-subunit rRNA gene of *E. histolytica* and then amplified by traditional and real-time PCR. Out of these 205 stool and liver abscess pus specimens, 124 were positive by the real-time-PCR assay and 90 were positive by the traditional-PCR test. Compared to the real-time-PCR assay, the antigen detection test was 79% sensitive and 96% specific. When the traditional-PCR test results were compared to the real-time-PCR assay, the sensitivity of traditional PCR was 72% and the specificity was 99%. In conclusion, all three methods for the detection of *E. histolytica* were highly specific, with real-time PCR being the most sensitive.

The World Health Organization has recommended that *Entamoeba histolytica* “should be specifically identified and if present should be treated” (27). Classic microscopic examination of the parasite *E. histolytica* in stool cannot differentiate it from the nonpathogenic but identically appearing parasites *Entamoeba dispar* and *Entamoeba moshkovskii*. While *E. histolytica* trophozoites are more likely than *E. dispar* and *E. moshkovskii* to contain ingested erythrocytes, most often *E. histolytica* trophozoites in patient stools lack ingested red cells (7, 10, 11). Not only is microscopy unable to differentiate *E. histolytica* from *E. dispar* or *E. moshkovskii*, it is at best only 10 to 60% sensitive and confounded with false-positive results due to misidentification of macrophages and nonpathogenic species of *Entamoeba* (12, 13, 18, 23). Culture along with isoenzyme (zymodeme) analysis enables differentiation of *E. histolytica* from *E. dispar* or *E. moshkovskii* and was considered the gold standard for diagnosing amebic infection in the last 2 decades. However, amebic cultures and isoenzyme analysis require a week to complete and are negative with many microscopy-positive stool samples, in some cases due to delay in sample processing or due to the institution of antiamebic therapy prior to stool collection (13, 14, 23).

New approaches to the detection of *E. histolytica* are based on the detection of an *E. histolytica*-specific antigen and DNA. Several groups have reported the detection of amebic antigen in stool samples, serum, liver abscess pus samples, and saliva using enzyme-linked immunosorbent assay methods (1, 2, 4, 5, 9, 12, 13, 14, 15, 16, 19, 20, 21, 22, 23). The TechLab (Blacksburg, Virginia) *Entamoeba histolytica* II kit is the only Food

and Drug Administration-approved test that is specific and sensitive for the detection of *E. histolytica* in feces (15). This antigen detection assay captures and detects the parasite’s Gal/GalNAc lectin from stool samples.

Several PCR-based tests that detect *E. histolytica*-specific DNA in stool samples and liver abscess pus have also been developed and evaluated (6, 12, 13, 24, 25, 28). The sensitivities and specificities of traditional PCR-based methods for the diagnosis of *E. histolytica* infection in stool samples were comparable to those of culture and antigen detection (8, 12, 15). Real-time PCR is a new methodology that employs fluorescent labels to enable continuous monitoring of amplicon (PCR product) formation throughout the reaction. Real-time-PCR assays for *E. histolytica* using TaqMan-based probes have been reported in the literature but have not been compared to the traditional-PCR test and antigen detection tests presently available for diagnosis (3, 26).

In this study a molecular-beacon-based real-time-PCR assay for the rapid detection of *E. histolytica* was evaluated using fecal and amebic liver abscess pus specimens. Results from the real-time-PCR assay were compared to the *E. histolytica*-specific antigen detection test as well as to an established traditional nested-PCR assay reported earlier.

MATERIALS AND METHODS

***E. histolytica* culture.** A laboratory isolate of *E. histolytica* cultured in Robinson’s xenic medium was sedimented by centrifugation and resuspended in phosphate-buffered saline, pH 7.2. For standard curves of the different diagnostic test results, the parasites were serially diluted 10-fold from a starting concentration of 100,000 trophozoites/ml.

Fecal and liver abscess pus specimens. Fecal specimens included in this study were from children in Mirpur, an urban slum in Dhaka, Bangladesh, who were participants in a prospective study of amebiasis (17). All samples were preserved at –70°C until analyzed. Intestinal amebiasis was defined by the presence of *E. histolytica* antigen in the stool sample detected by the antigen detection test. A total of 182 fecal specimens from 125 children aged 6 to 10 years were used in this study. Out of these 182 stool specimens, 108 were nondiarrheal stool specimens

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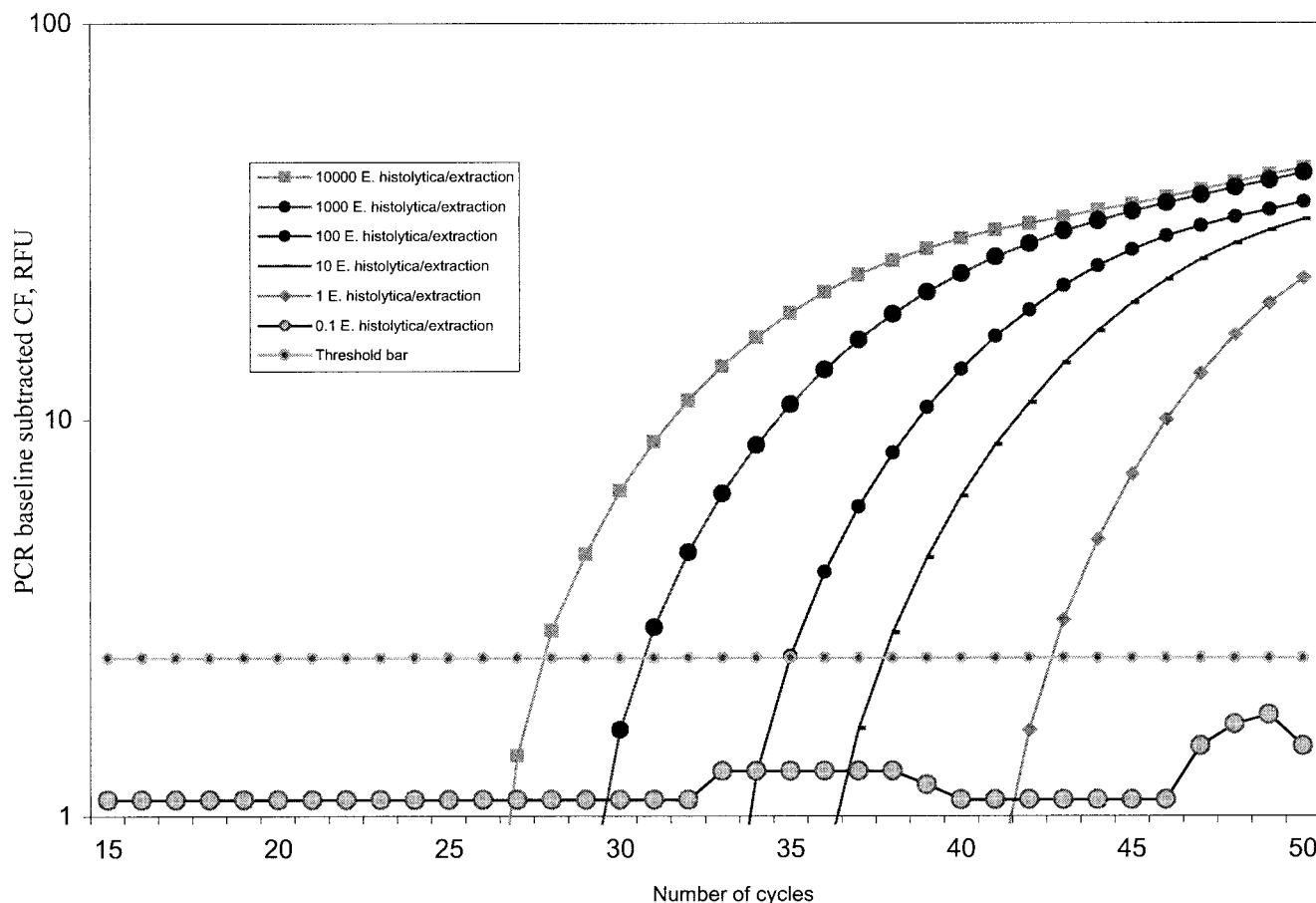


FIG. 1. Analytic sensitivity of the *E. histolytica* real-time-PCR assay. Shown are levels of amplification of *E. histolytica* organisms from human feces containing parasites numbering 10^4 , 10^3 , 10^2 , 10, 1, and 0.1 per extraction.

collected from 82 children as part of routine surveillance. Seventy-four fecal specimens were collected from 53 children during acute episodes of diarrhea. Out of the total of 182 stool samples, 84 fecal specimens were positive by the TechLab *E. histolytica* II antigen detection test and 98 were negative. Amebic liver abscess was defined clinically as a space-occupying lesion in the liver of a patient with a positive serum antiamebic antibody test (17). Liver abscess pus was aspirated only for clinical purposes as judged by the clinicians caring for these patients and not for the purpose of this study. Out of the 23 liver abscess pus samples examined, 17 were positive by the TechLab *E. histolytica*-specific antigen detection test.

Informed consent was obtained from the parents or guardians of the children, and the human experimentation guidelines of the U.S. Department of Health and Human Services, the University of Virginia, and the Centre for Health and Population Research of the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh, were followed in the conduct of this research.

Antigen detection test. The TechLab *E. histolytica* II test (designed to detect specifically *E. histolytica*) was performed on stool specimens according to the manufacturer's instructions. Liver abscess pus specimens were vortexed and then centrifuged at 14,000 rpm for 5 min, and 100 μ l of the resulting undiluted supernatant was used for antigen detection. A test was considered positive when the optical density reading of a sample at 450 nm was ≥ 0.15 .

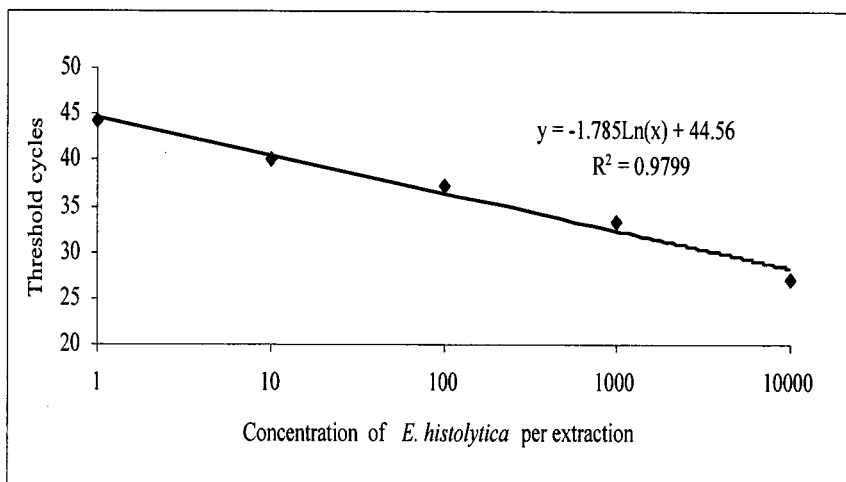
Extraction of DNA from fecal or liver abscess pus specimens. Fecal or liver abscess pus specimens (0.2 g) were used for DNA extraction. The specimens were washed twice with sterile phosphate-buffered saline and centrifuged for 5 min at 14,000 rpm. DNA was extracted using the QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions except that the suspension was incubated in the kit's stool lysis buffer at 95°C and a 3-min incubation with the InhibitEx tablets was used. The DNA was eluted in 0.2 ml AE buffer (supplied with the QIAGEN kit). Genomic DNA was obtained by the same method from *E. dispar* strain SAW760, the *E. moshkovskii* Laredo

strain (both obtained from the London School of Hygiene and Tropical Medicine, London, United Kingdom), and *Blastocystis hominis* (our own laboratory strain).

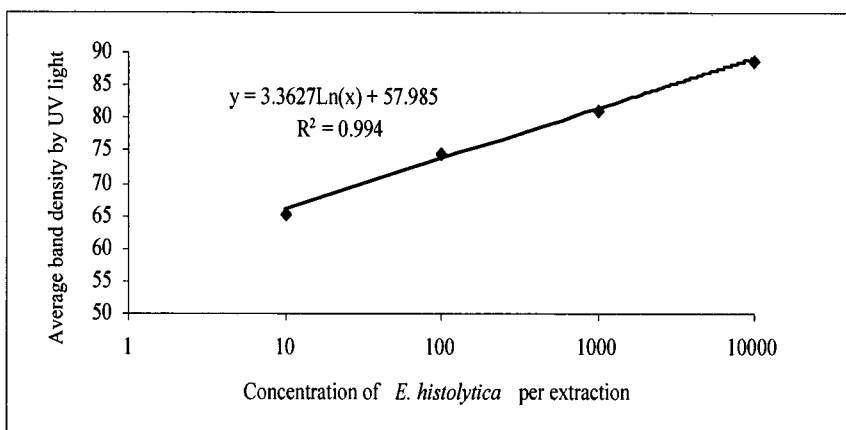
Traditional-PCR assay. Traditional nested PCR for the detection of *E. histolytica* in fecal and liver abscess pus specimens was carried out according to a protocol previously described (12). The assay was based on the amplification of the small-subunit rRNA gene of *E. histolytica*. The PCR products were analyzed in 1.2% agarose gels, and the average band densities of the PCR products were measured using Quantity One software (Bio-Rad).

Real-time-PCR assay. The oligonucleotide primers and molecular-beacon probe were designed (using Oligo software from Beacon Designer-2.1; Bio-Rad) to specifically amplify a 134-bp fragment inside the 16S-like small-subunit rRNA gene of *E. histolytica* (Gene Bank accession number X64142). Primers and probe were purchased from Eurogentec, United Kingdom. The *E. histolytica*-specific primers and probe set consisted of the forward primer (Ehf) 5'-AAC AGT AAT AGT TTC TTT GGT TAG TAA AA-3' and the reverse primer (Ehr) 5'-CTT AGA ATG TCA TTT CTC AAT TCA T-3'. The molecular-beacon probe used for this assay was a double-labeled probe, Texas Red-GCAGC-ATT AGT ACA AAA TGG CCA ATT CAT TCA-GCTCGC-dR Elle. The underlined six bases at the 5' and 3' ends of the probe were additional sequences required to form a hairpin loop.

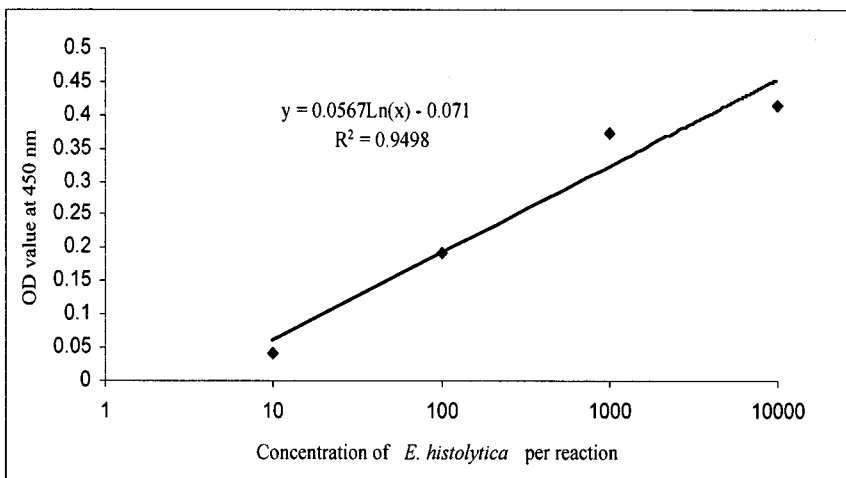
Amplification reactions were performed in a volume of 25 μ l with Bio-Rad's IQ super mix (100 mM KCl; 40 mM Tris-HCl, pH 8.4; 1.6 mM deoxynucleoside triphosphates; *iTaq* DNA polymerase, [50 units/ml; 7.5 mM MgCl₂]), 25 pmol of the Ehf and Ehr primers, 6.25 pmol of the *E. histolytica*-specific molecular-beacon probe, 7.75 μ l of distilled H₂O, and 2.0 μ l of the DNA sample. Amplification consisted of 45 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 15 seconds at 72°C. The ramping of the machine was 3.3°C/second in every step. Amplification, detection, and data analyses were performed with the i-Cycler real-time detection system (Bio-Rad). Fluorescence at 575 nm was measured



A



B



C

FIG. 2. Comparisons of the standard curves for real-time PCR, traditional nested PCR, and antigen detection. (A) Plot of mean C_T values for *E. histolytica* by real-time PCR. The plot of C_T values and DNA input fits a linear function ($R^2 = 0.979$). (B) Plot of average band density by traditional PCR. The plot of average band density and DNA input fits a linear function ($R^2 = 0.994$). (C) Plot of mean optical density values at 450 nm by the antigen detection test. The plot of optical density values and concentrations of *E. histolytica* input organisms fits a linear function ($R^2 = 0.949$).

TABLE 1. Comparison between the real-time PCR assay and the *E. histolytica*-specific antigen detection test

Real-time-PCR result	No. of specimens with antigen detection test result		
	Positive	Negative	Total
Positive	98	26	124
Negative	3	78	81
Total	101	104	205

during the annealing step of each cycle. The performance of the assay was evaluated using a range of control samples including *E. dispar*, *E. moshkovskii*, and *B. hominis*. Amplification results were analyzed using i-Cycler software, version 3.0 for Windows. Amplification was also confirmed in all reactions by gel electrophoresis. A sample was considered positive if the signal cycle threshold (C_T) value exceeded a preset threshold.

Analytical sensitivity and specificity using cultured organisms. To establish the minimum number of parasites detectable by the *E. histolytica*-specific real-time molecular-beacon assay (detection limit), serial dilutions of cultured trophozoites of *E. histolytica* were used. To estimate the analytical specificity of the *E. histolytica* real-time molecular-beacon assay, we tested DNA from other entamebae, including *E. dispar* and *E. moshkovskii*, as well as *Giardia lamblia* and *Blastocystis hominis*.

Sensitivity and specificity of real-time PCR using clinical specimens. The sensitivity and specificity of the *E. histolytica*-specific real-time molecular-beacon assay were calculated with 205 fecal and liver abscess pus specimens using the *E. histolytica*-specific antigen detection test as the gold standard because of its superior performance over microscopy, culture, and traditional PCR (17, 19, 20). Sensitivity was calculated as the number of true positives divided by the sum of true positives and false negatives, and specificity was calculated as the number of true negatives divided by the sum of true negatives and false positives. The sensitivities and specificities of all three methods were also evaluated against a combined gold standard in which any specimen positive by one of these three assays was considered positive.

RESULTS

Real-time-PCR sensitivity and specificity. The amplification plot of the *E. histolytica*-specific real-time-PCR assay is shown in Fig. 1. The analytical sensitivity of the developed real-time-PCR assay was evaluated using cultured trophozoites of *E. histolytica* that were serially diluted in phosphate-buffered saline buffer. The detection limit for the real-time-PCR assay was 10 trophozoites of *E. histolytica* per milliliter (Fig. 2A). Traditional PCR had a lower level of detection of 100 parasites per milliliter (Fig. 2B), while the antigen detection test required 50 parasites per reaction (Fig. 2C). The real-time-PCR assay was specific for *E. histolytica* detection, as it was negative when DNA was introduced from other *Entamoeba* species, including *E. dispar* and *E. moshkovskii*, as well as from *Giardia lamblia* and *Blastocystis hominis* (data not shown).

TABLE 2. Comparison of the real-time-PCR assay and the traditional PCR for the detection of *E. histolytica*

Real-time-PCR result	No. of specimens with traditional-PCR result		Total
	Positive	Negative	
Positive	89	35	124
Negative	1	80	81
Total	90	115	205

TABLE 3. Mean and median C_T values of real-time-PCR assay according to the antigen detection test and traditional PCR positivity

Category	No. of specimens	C_T value	
		Mean	Median
Antigen detection test positive and real-time-PCR positive	98	35.6	36.3
Antigen detection test negative and real-time-PCR positive	26	40.3	43.1
Traditional-PCR positive and real-time-PCR positive	89	34.4	34.3
Traditional-PCR negative and real-time-PCR positive	35	42.1	43.4
Antigen detection test negative and traditional-PCR negative	21	42.7	43.5

Comparison of real-time-PCR, antigen detection, and traditional-PCR assays on stool and liver abscess pus specimens.

The results of the antigen detection test, traditional nested PCR, and real-time PCR for 205 stool and liver abscess pus specimens are presented in Tables 1 and 2. Of these 205 specimens, 124 were positive by real-time PCR, 101 by antigen detection, and 90 by traditional nested PCR. For the 101 specimens that were positive by the antigen detection test, the real-time PCR was positive for 98 specimens, for a sensitivity of 97%. In contrast, compared to real-time PCR, the antigen detection test was 79% sensitive and 96% specific. The correlation between these two tests was 86%. Out of the 90 specimens that were positive by the traditional nested PCR, the real-time PCR was positive for 89 specimens, for a sensitivity of 98%. Compared to real-time PCR, the traditional PCR was 72% sensitive and 99% specific. The correlation between these two tests was 82%. The correlation between the antigen detection test and the traditional PCR was 90% (data not shown). Real-time PCR was positive in 20/23 liver abscess pus specimens, with the 3 negative specimens from samples collected from patients who had already received antiamebic therapy for 8 days (one patient) and 30 days (two patients). Antigen detection was positive for only 17/23 liver abscess pus specimens. Compared to a combined gold standard defined as any one of the tests being positive for *E. histolytica*, the sensitivities of real-time PCR, the antigen detection test, and traditional PCR were 98%, 80%, and 71%, respectively.

The semiquantitative nature of real-time PCR may allow for an estimation of parasite load in the different clinical samples. There was no significant difference between the C_T values from nondiarrheal and diarrheal stool specimens. However, the mean C_T value of the liver abscess pus specimens (37 ± 0.60) was significantly higher than that of the stool specimens (35 ± 0.91) ($P = 0.02$). Specimens that were negative by the antigen detection test or traditional PCR but positive by real-time PCR had higher mean and median C_T values, consistent with the real-time-PCR assay having a higher sensitivity than the other two tests (Table 3).

DISCUSSION

In this study we developed and evaluated a real-time molecular-beacon PCR assay for the detection of *E. histolytica* from fecal specimens. The main advantages of this real-time-PCR assay over the traditional nested-PCR test were that (i) it

requires only one PCR step, compared to at least two in the traditional nested PCR; (ii) it is performed in a closed system where post-PCR handling is not required; and (iii) the assay is highly sensitive and could be used for quantitative purposes. The sensitivity of this real-time-PCR assay was comparable to that of the TaqMan-based test reported by Blessmann et al. (3). However, in contrast to Blessmann et al., we compared the sensitivity of real-time PCR to those of antigen detection and traditional PCR and extended its use to the analysis of liver abscess pus specimens.

Our results with cultured trophozoites of *E. histolytica* clearly indicate that the real-time-PCR assay developed in this study is more sensitive for the detection of *E. histolytica* than traditional PCR or antigen detection. The analytical sensitivity of this real-time-PCR assay is around 0.02 parasite per reaction, which is around 1 parasite in the specimen that will be extracted for DNA. The real-time-PCR assay detected almost all of the positive specimens detected by the antigen detection test and the traditional-PCR test. Compared to a gold standard defined as any one test being positive, the real-time-PCR test exhibited superior sensitivity. The specificities of the antigen detection test and traditional-PCR test compared to that of the real-time PCR were excellent (Tables 1 and 2). The higher C_T values of the specimens that were positive by real-time PCR but negative by the antigen detection test and traditional-PCR test indicate that the low number of parasites in those samples fell below the detection limits of the antigen detection test and traditional PCR. Due to the excellent specificity of these three methods, the choice of assay is likely to depend on the expertise, need, and equipment available in the laboratory. Now that sensitive and specific diagnostic tests are available to study the epidemiology of amebiasis, it is important to have more accurate data on the prevalence of *E. histolytica* in various part of the world to estimate the burden of this disease.

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