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Short Communication

Leishmaniasis in Turkey: Determination of *Leishmania* Species by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

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Abstract

Background: Cutaneous leishmaniasis (CL) is endemic in Southeastern Anatolia, mainly in Sanliurfa and Hatay provinces, and the causative agents are mostly *Leishmania tropica* and less frequently *L. infantum*. Here, we report the first MALDI-TOF analyses of *Leishmania* promastigotes obtained from the cultures of two CL cases from Osmaniye and Hatay provinces who were initially diagnosed by microscopy, culture and identified as *L. infantum* with Real-Time PCR (RT-PCR).

Methods: Samples obtained from the skin lesions of patients were initially stained with Giemsa and cultivated in NNN medium. Examination of the smears and cultures revealed *Leishmania* amastigotes and promastigotes, respectively. The promastigotes (MHOM/TR/2012/CBU15 and MHOM/TR/2012/MK05) obtained from the cultures of both patients were used for RT-PCR targeting the ITS-1 region in the SSU of rRNA. The reference strains of four *Leishmania* species (*L. infantum, L. donovani, L. tropica* and *L. major*) were initially assessed with MALDI-TOF and their data were added to MALDI-TOF Biotyper Library.

Results: Both RT-PCR and MALDI-TOF analyses indicated that the causative agent in both patient samples was *L. infantum*.

Conclusion: Despite disadvantages such as requirement of culture fluid with nothing but promastigotes and high cost, MALDI-TOF analysis may be a fast, sensitive and specific diagnostic tool in especially large-scale research studies, where the cost declines, relatively.

Introduction

Leishmaniasis is a common vector-borne parasitic disease with wide spectrum of clinical forms, ranging from relatively mild cutaneous infection to life-threatening visceral disease. It is transmitted by sand flies (*Phlebotomus spp.* and *Lutzomyia* spp.), and according to World Health Organization (WHO), almost 12 million people from 98 countries or regions worldwide are infected with leishmaniasis (1, 2).

Leishmaniasis is a significant parasitic disease in Turkey, as well. Common causative agents of leishmaniasis in Turkey are: *Leishmania infantum* in visceral disease, which is sporadic in Aegean, Mediterranean, and Central Anatolia; *L. tropica* or *L. infantum* in cutaneous infections, which are common in southeastern and Mediterranean regions (1, 3-6). According to the data of the Ministry of Health, 46.003 new CL cases have been reported in Turkey between 1990 and 2000, 96% of whom were from provinces located in the southeastern Anatolia (7). In the last decade, there has been a significant elevation in the number of leishmaniasis cases and focuses in many provinces in Aegean and Mediterranean Regions, which indicate that leishmaniasis may pose an emerging threat to public health in Turkey (3, 5, 7-9).

The diagnosis of leishmaniasis relies primarily on microscopic examination of bone marrow aspiration samples or cultured material in a specific medium such as NNN and RPMI. Serology and molecular methods such as polymerase chain reaction (PCR) are also used in the diagnosis (1). In a study conducted in southern Turkey, PCR was found to be more sensitive and specific in the diagnosis of leishmaniasis, compared to microscopy and culture (5).

Microbial identification procedures based on the determination of species-specific peptides and proteins by using mass spectrophotometers were introduced almost 30 years ago (10). Recently, a new technology called "soft-ionization mass spectrometry", which facilitated the examination of biomolecules without disintegration or with disintegration after the application of High Performance Liquid Chromatography (HPLC) and/or electrophoresis was introduced (11). It is expected that improvement of this method may enhance its use for rapid and correct identification of microbial agents in human infections (12-14).

MALDI-TOF (Matrix-assisted Laser Desorption Ionisation - Time of Flight Mass Spectrometry) is also a new diagnostic method currently used in microbiology laboratories for rapid and reliable identification of bacteria and yeasts. The application of this method is easy; initially, the clinical specimen is ionized in a specific acidic solution (matrix) using laser beams. This is followed by evaporation towards the sensor in the equipment. The "time of flight" (TOF) depends on the molecular weight of the ionized molecules. The development of faster instruments with higher mass accuracy and sensitivity, the implementation of new lasers, the advent of advanced TOF/TOF instruments, as well as the introduction of new MALDI matrices have all improved the technique and widened its application range (15). Today, it is relatively fast; the total procedure may take almost 20 minutes. It is also highly effective in the identification of bacteria in pure cultures (16, 17).

Assessments with MALDI-TOF rely on the reference strains of microbiological agents present in the database. Identification of an isolate during an assessment is based on the proximity to a reference strain in the database. Compared to conventional identification methods, more bacterial isolates (over 95%), including gram-positives, enterobactericea, mycobacteria and anaerobic bacteria, can be identified with MALDI-TOF at species level (17-19).

Here, we present two CL cases from southeastern Turkey diagnosed initially with mi-

croscopy and culture, and then found to be infected with *L. infantum*, the relatively rare agent for CL in the region. The presence of *L. infantum* was finally demonstrated for the first time using MALDI-TOF MS at a reliable level (score: > 2000) in a very short time.

Material and Methods

The Leishmania reference strains

The isolates of *L. infantum* (MHOM/TN/1980/IPT1), *L. donovani* (MHOM/IN/1980/DD8), *L. tropica* (MHOM/AZ/1974/SAF-K27) and *L. major* (MHOM/SU/1973/5ASKH), used as the international reference controls in the study, were kindly provided by Professor Charles Jaffe from the Department of Parasitology of Hebrew University Hadassah Medical School in Israel.

Patients

Both patients were admitted to Mustafa Kemal University School of Medicine Department of Parasitology. Informed consents of both patients were received and the present study was approved by the Ethics Committee of Celal Bayar University Medical Faculty, prior to study.

- **Patient No 1:** Nineteen year old, male, farmer from Hatay province. He had one long-standing (almost 1 year old) lesion on his right cheek. He reported having close contact with cattle (Figure 1a).
- **Patient No 2:** Twenty-one year old, male, farmer from Osmaniye province, having one long-standing (more than a year and a half) lesion on his forearm (Figure 1b). He reported that he had been living in the village since he was born, and there was a stable near his house with cattle and many dogs. He was initially diagnosed as CL in August 2011 in Osmaniye and given treatment. However, he reported that he missed the follow-up visits after treatment, and the infection relapsed within 6 months after the initial diagnosis.

Diagnosis

Following the clinical diagnosis, lesion samples were obtained for parasitological diagnosis with microscopy and culture. The personal data and the lesion history of both patients were recorded together with the clinical features on the day of physical examination.

Microscopy

Three smears were prepared from the lesion samples of each patient. These smears were initially fixed with methanol and stained with Giemsa. Smears were then examined using a light microscope under x1000 magnification.

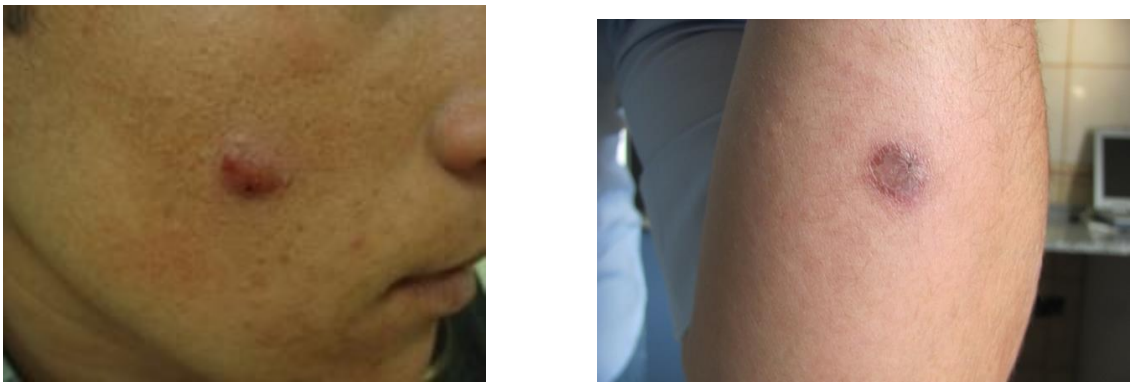

Fig. 1: Lesions due to cutaneous leishmaniasis infection on patients a) right cheek of the first patient (left; MHOM/TR/2012/MK05 HATAY); b) left forearm of the second patient (right; MHOM/TR/2012/CBU15 OSMANIYE)

Culture

Aspiration material was taken from the lesion using a fine needle and inoculated immediately in Nicolle-Novy-McNeal (NNN) culture medium. The culture tubes were kept in 24°C, and after 7 days of culture, the *Leishmania* promastigotes were observed and transferred to the flasks containing 5 ml of RPMI-1640 medium, 10% of foetal calf serum, 200 U/ml of penicillin and 0.2 mg/ml of streptomycin, and kept at 25°C for mass cultivation. The tubes containing promastigotes were checked once in every 3 days to detect any reproduction. The final concentration was adjusted to $1x10^8$ promastigotes/ml.

DNA Isolation

DNA isolation of lesion samples and smears was done using "Roche High Pure PCR Template Preparation Kit". The DNAs were then kept at -20^0C until use in PCR.

ITS1 RT-PCR

A Real-Time PCR procedure which targeted the genes that encode SSU rRNA and 5.8S rRNA in the internal transcribed spacer (ITS) -1 region of *Leishmania* parasites was used for the assessments, as previously described (20). The primers (Forward: 5'-GCCGATCGACGTTGTAG-3'; Reverse: 5'-GGAAGCCAAGTCATCCAT-3') and probes (Probe 1: LC Red 640–ACCGAAACGCCGTCTATTTTTTTTG-Phosphate; Probe 2: CCTCTCTCTCCCTCCCGCCAA - Fluorescein) were selected specifically for the procedure. The reaction mixture (10 µl) included 1.5 µl H_20 (PCR grade water-Roche Applied Science®), 0.8 µl $MgCl_2$ (25 mM), 1 µl Forward Primer, 1 µl Reverse Primer, 1 µl Probe1, 1 µl Probe2, 1 µl LightCycler FastStart DNA Master Mix (Roche Applied Science®) and finally 2.7 µl of genomic DNA.

The thermal profiles of each parasitic agent (*L. tropica, L. donovani, L. infantum* and *L. major*) consisting of denaturation, amplification, melting curve analysis and cooling steps, were identified individually and recorded in the LightCycler®. The reaction mixtures were transferred in capillary tubes of the LightCycler®at 20 µl after centrifugation at 2000 RPM at 10 seconds (21).

Sample preparation for MALDI-TOF analysis

The *Leishmania* isolates recovered in RPMI medium were used in MALDI-TOF analysis. Initially, 10 millilitres of culture were centrifuged at 1500 RPM for 10 minutes, the supernatant was discarded and 10 ml of distilled water was finally added to tubes. This was repeated once again. Then, the sediment was taken to a new tube, and 300 µl of distilled water was added together with 900 µl of ethanol and mixed thoroughly. The tubes were centrifuged at 14500 RPM for 5 minutes and the supernatant was discarded. The pellet was left for drying in the air. Then, 20 µl of 70% formic acid was added to the tubes and the tubes were vortexed before the same amount of acetonitrile was added. The tubes were centrifuged at 14500 RPM once again for 5 minutes and the supernatant was discarded. Finally, 1 µl of supernatant was loaded onto the polished steel MALDI target plate (Bruker Daltonics®, Bremen, Germany) and allowed to dry at room temperature. The samples were overlaid with 1 µl of matrix solution which contained saturated α-cyano-4-hydroxycinnamic acid in a solution of 50% of acetonitrile and 2.5% of trifluoroacetic acid and the plate was air-dried at room temperature. The plate was then placed in Bruker® Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics®; Bremen, Germany). The spectra were automatically recorded in the linear positive ion mode with delayed extraction at a laser frequency of 20 Hz within a mass range from 4000 to 10000 Da. A total of 600 satisfactory shots in 100 shot steps from the sampling area of the target spot were obtained for each spectrum. The spectra were eligible for further analysis when the peaks had a resolution degree better than 400 Da. Each run included a test standard with a characteristic peptide and protein profile, provided by

Bruker Daltonics® for calibration, a negative extraction control and the international reference *Leishmania* strains (MHOM/AZ/19-74/SAF-K27, MOHOM/SU/1973/5ASKH, MHOM/TN/1980/IPT1 and MHOM/IN/19-80/DD8) used in the study. If the test gave no results, the assay was conducted once again according to the same procedures.

Data Analysis

The mass spectra were evaluated with FlexAnalysis software version 3.0 (Bruker Daltonics®, Bremen, Germany). The automated data analysis was processed with MALDI Biotyper software version 3.0 (Bruker Daltonics®, Bremen, Germany). The list of the best peaks of the spectrum was created automatically by the software after smoothing, normalization and baseline subtraction.

Addition of reference strains of four Leishmania species to Maldi Biotyper Library (MBL)

Four reference strains of *Leishmania* species (*L. tropica*, *L. major, L. infantum* and *L. donovani*) were added to MBL for the first time during the present study (Figure 2). The spectra of these four *Leishmania* species were used to supplement the database of the standard MBL; thus, the supplemented MBL (now referred as s-MBL) consisted of MBL and the spectra of four reference strains of *Leishmania* species.

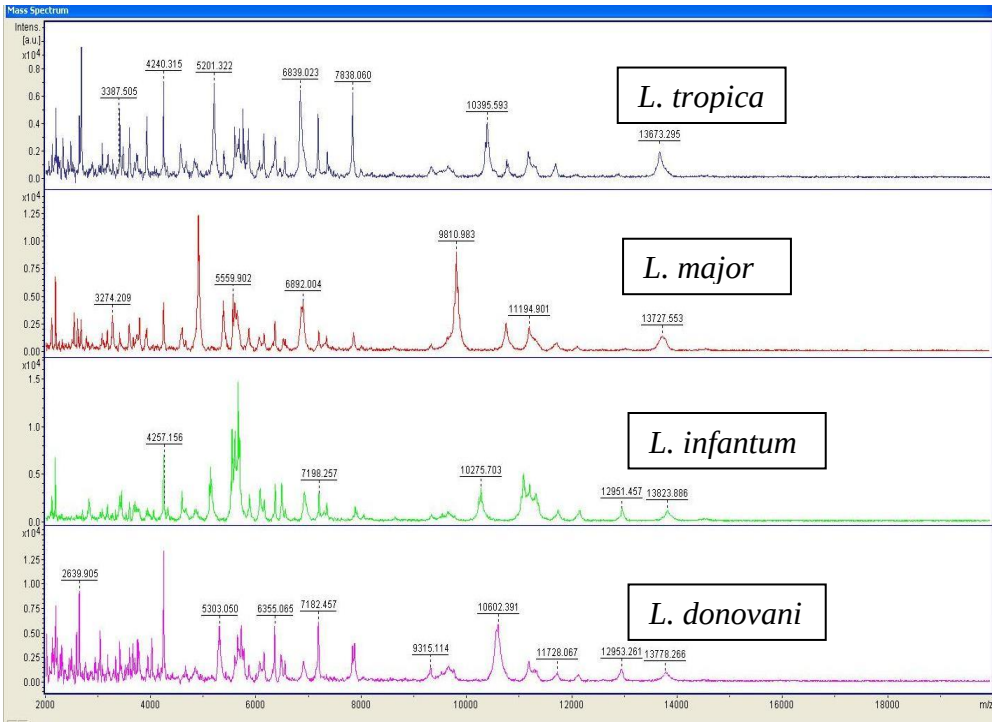

Fig. 2: MALDI-TOF profiles of the reference strains of four *Leishmania* species.

Evaluation of the Results

The spectra of the patients were analyzed by standard pattern matching algorithm, which compared the raw spectra with the spectra of the MBL and the results were listed in a ranking table. The results were expressed as log (score) values, which ranged from 0 to 3. Generally, the score values over 1.7 indicated relationships at the genus level, while the values over 2.0 indicated relationships at the species level. The highest score obtained during

the analysis was used for the identification of species.

Results

Examination of the Giemsa-stained smears and culture samples of the patients revealed *Leishmania* amastigotes and promastigotes, respectively. Further analyses with ITS-1 RT-PCR using melting curve analyses demonstrated that the causative agent was *L. infantum* in both cases (Fig. 3).

The spectra of both patients in MALDI-TOF analysis were found to be concordant with *L. infantum*, as well, with a log (score) value over 2.000 m/z (Fig. 4).

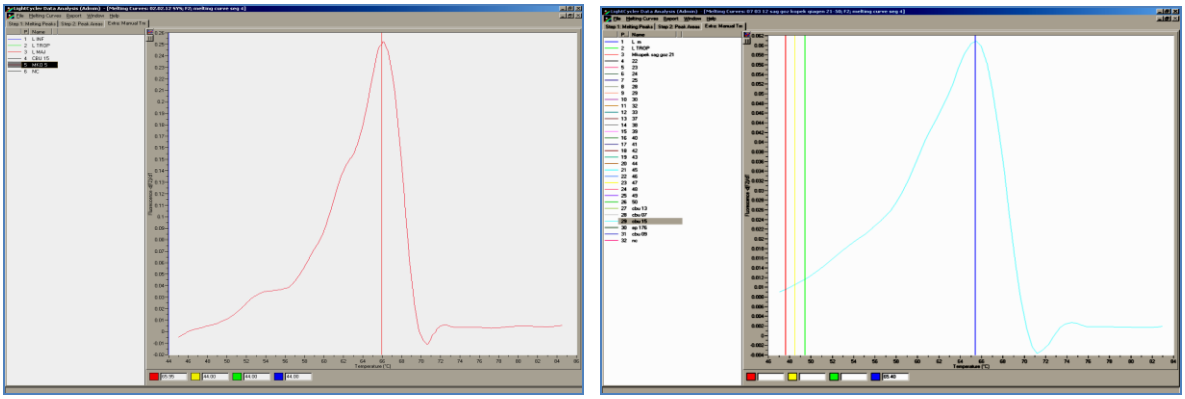

Fig. 3: Melting curve analyses of both patients revealed curves concordant with *Leishmania infantum*

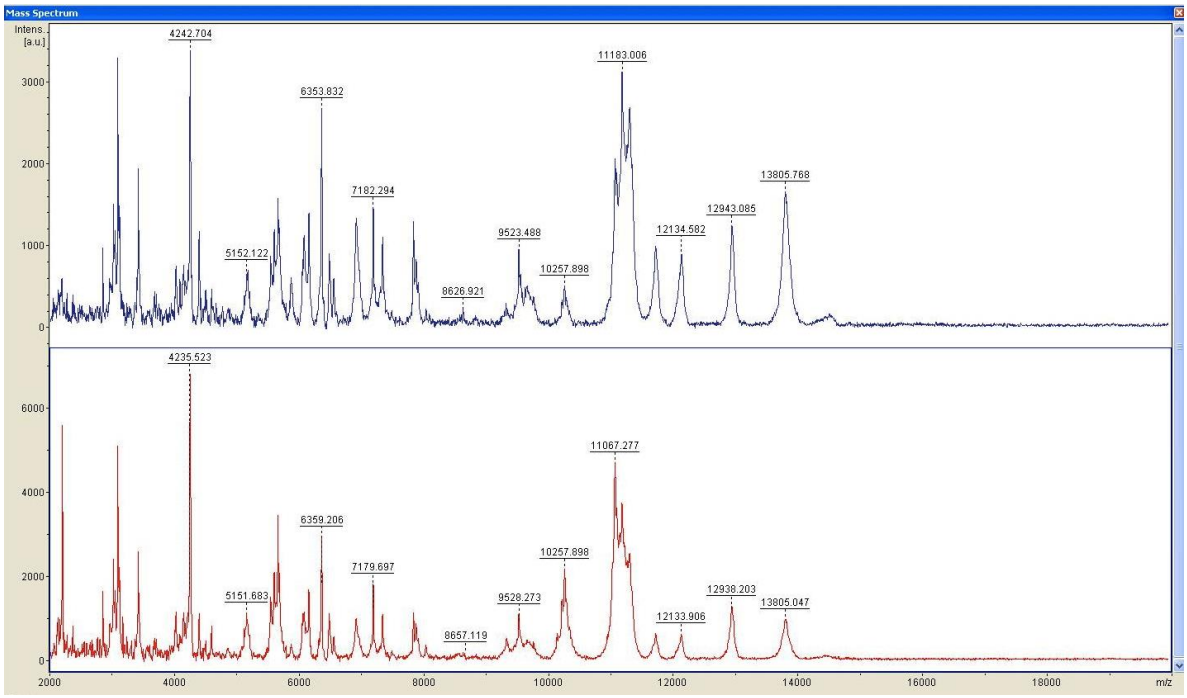

Fig. 4: MALDI-TOF profiles of two *Leishmania* isolates obtained from patient 1 (upper) and Patient 2 (lower)

Discussion

The conventional method for species identification of *Leishmania* isolates is multilocus enzyme electrophoresis (MLEE) (22). Yet, different PCR/PCR-RFLP based methods targeting the genes encoding kinetoplastic DNA, telomeric sequences, gp63, miniexons, β-tubulin, or ribosomal RNA (particularly the internal transcribed spacers, ITS) and recently the microsatellites, have also been proposed for the identification of *Leishmania* species (23). The ITS-1 region of the ribosomal DNA repeat unit (rDNA-ITS1) has previously been exploited for the discrimination of Old World *Leishmania* species using RFLP (24-26), reverse hybridization assays (27) and melting curve analysis (28). Advances made in clinical proteomic technologies have further enhanced our mechanistic understanding of bio-pathological features of *Leishmania* species, which lead us to define novel targets suitable for vaccine development. This is useful for both identification and characterization of the defined leishmanial antigens, and elucidation of the range and specificity of anti-leishmanial immune responses in the body (29).

Recently, successful use of two-dimensional electrophoresis (2-DE) has been reported for the identification of *L. infantum* (30). Indeed, these researchers described MALDI-TOF Mass Spectra characterization and theoretical study of the Peptide Mass Fingerprints (PMFs) of a new protein sequence, as well. Their work focused mainly on MASCOT, BLAST alignment, and alignment-free QSAR prediction of the contribution of 29 peptides found in the PMF of the new protein to specific enzyme action (30). This combined strategy may be used to identify and predict peptides of prokaryotic and eukaryotic parasites and their hosts as well as other superior organisms, which may be of interest in target identification or drug development against infectious agents. In another study, Gupta and his colleagues reported that using 2-DE, MALDI-TOF and MALDI-TOF/TOF-MS, they identified a total of 33 proteins belonging to *L. donovani* (31). They concluded that proteomics may be regarded as a method of choice in characterizing a complex protein fraction (F2) of soluble *L. donovani* promastigote antigens, which may further be vaccine candidates against visceral leishmaniasis. Gupta and his colleagues also presented the data of another proteomic study focusing on completely unknown or hypothetical proteins of *L. donovani*, which may represent novel potential targets for drug development or putative vaccine candidates (31).

Proteomic analyses of *Leishmania* species have been applied to study the developmental differentiation of parasites. Early reports on the *L. infantum* proteome identified over 2,000 spots with 2-DE, and the comparison between promastigote and axenic amastigote patterns of *Leishmania spp.* revealed almost 3% of differentially-expressed spots (32). Such a difference was also reported for the axenic amastigotes of *L. donovani* (5%) and *L. mexicana* (7%) (33). Comparative proteomic analysis of *Trypanosoma cruzi*, a close relative of *Leishmania* spp., has uncovered interesting information on the energy sources used in different life stages of the parasites (34). However, large-scale identification of protein spots of *Leishmania* species has yet to be identified.

There has been a growing interest in the identification of parasitic species using MALDI-TOF MS. Initially, it has been used in a study for the identification of pyrimethamin resistance in *Plasmodium falciparum isolates* (35). Recently, MALDI-TOF MS was shown to be effective in the differentiation of *Blastocystis* subtypes in axenic cultures (36). Concerning *Leishmania* species, two studies have recently been published. Cassagne and colleagues have identified 66 of 69 *Leishmania* isolates of patients with MALDI-TOF at reliable levels; this indicates that MALDI-TOF may be used effectively for the identification of *Leishmania* isolates at species level (37). In a study from India, researchers identified soluble proteins of *L. donovani*, which are potential drug targets

after assessments with 2D electrophoresis and MALDI-TOF (38).

Conclusion

Our study is the first report of the application of MALDI-TOF MS for the identification of *Leishmania* species isolated in Turkish patients. Enrichment of the Maldi-Biotyper Library (MBL) for *Leishmania* species and other parasitic agents that may cause human infections will obviously contribute to quick and reliable identification of the species of parasitic agents that may cause severe epidemics worldwide, especially in developing countries.

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