Use of FTA Cards for Direct Sampling of Patients’ Lesions in the Ecological Study of Cutaneous Leishmaniasis\V

Hirotomo Kato,1* Abraham G. Cáceres,2,3 Tatsuyuki Mimori,4 Yuka Ishimaru,1 Amal S. M. Sayed,1,5 Megumi Fujita,1 Hiroyuki Iwata,3 Hiroshi Uezato,9 Lenin N. Velez,2 Eduardo A. L. Gomez,3,9 and Yoshihisa Hashiguchi4

Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan;2 Sección de Entomología, Instituto de Medicina Tropical Daniel A. Carrion, Facultad de Medicina, Universidad Nacional Mayor de San Marcos;3 and Laboratorio de Entomología, Instituto Nacional de Salud,3 Lima, Peru;4 Department of Microbiology, School of Health Sciences, Kumamoto University, Kumamoto, Japan;5 Department of Animal Hygiene and Zoonoses, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt;6 Department of Dermatology, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan;7 Departamento de Leishmaniasis7 and Departamento de Oncocercosis, Servicio Nacional de Erradicacion de la Malaria (SNEM),8 Ministerio de Salud Publica, Guayaquil, Ecuador; and Department of Parasitology, Kochi Medical School, Kochi University, Kochi, Japan9

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The FTA card (Whatman) was assessed for its utility as a molecular epidemiological tool in collecting samples from patients with leishmaniasis in Peru because the card has a variety of merits; it is less invasive for patients and easy to handle for both physicians and other medical personnel for sample collection or diagnosis, in addition to its simplicity and easy countrywide and/or intercountry transportation for analysis. Samples were collected from 132 patients suspected of having leishmaniasis, and Leishmania species were successfully identified in samples from 81 patients in 15 departments of Peru by cytochrome b and mannose phosphate isomerase gene analyses. Of these, 61.7% were identified as Leishmania (Viannia) peruviana, 22.2% as L. (V.) braziliensis, 12.3% as L. (V.) guyanensis, 2.5% as L. (V.) shawi, and 1.2% as L. (V.) lainsoni. The three predominant species, L. (V.) peruviana, L. (V.) braziliensis, and L. (V.) guyanensis, were mainly found in the Andean highlands, in the tropical rainforest, and in northern and central rainforest regions, respectively. This is the first time L. (V.) shawi has been identified outside Brazil. The present study showed that the FTA card will be a useful tool for the ecological study of different forms of leishmaniasis. Furthermore, collecting samples directly from patients’ lesions by using the FTA card eliminates (i) the possibility of contamination of Leishmania isolates during short- and/or long-term passages of culture in vitro in each laboratory and (ii) pain and suffering of patients from taking samples by skin biopsy.

Leishmaniasis is caused by protozoan parasites of the genus Leishmania, which is further divided into two subgenera, Leishmania (Leishmania) and Leishmania (Viannia) (10). The disease is widely distributed around the world, especially in tropical and subtropical areas, affecting at least 12 million people in 88 countries (6). Approximately 20 Leishmania species are known to be pathogenic to humans, and the species is the major determinant of clinical outcome (cutaneous, mucocutaneous, and visceral forms) (6). Therefore, identification of the parasite species in areas of endemicity is important for both appropriate treatment and prognosis.

The standard method for the classification of Leishmania species is multilocus enzyme electrophoresis (MLEE), which requires the isolation and mass culture of the parasites (4, 17). This process has several disadvantages: (i) risk of contamination with bacteria and/or fungus and even other Leishmania isolates in the laboratory; (ii) maladaptation of the parasites to the artificial medium; (iii) difficulty in cultivation due to low numbers of parasites in cutaneous lesions, especially for the subgenus Leishmania (Viannia); and (iv) the time, several weeks or months, required to obtain a result after sample collection. All of these factors affect epidemiological studies in spite of considerable efforts to collect patient specimens from different areas of endemicity, especially for specimens from remote locations. To overcome these problems, molecular biological methods have been developed for the detection and identification of Leishmania species using DNA extracted from clinical samples (7, 16, 21). However, sampling procedures, such as skin biopsy, are sometimes painful for patients and become a burden to both patients and physicians. Therefore, alternative sampling procedures with less invasiveness, simple and easy handling, and greater convenience are required for the detection and identification of Leishmania species and continuous monitoring of endemic species of causative organisms.

FTA technology (Whatman) is a rapid and safe method for extracting nucleic acids from blood, cell, and pathogen samples without using any organic solvent or specialized equipment. When the samples are spotted onto an FTA card, the cells are readily lysed and the nucleic acids are fixed on the card, resulting in protection from nuclease, oxidative, and UV damage and prevention of the growth of bacteria and other microor-
organisms. The card is also suitable for long-term storage and the transportation of materials at room temperature, eliminating the possibility of contamination from isolates in vitro during the laboratory phase. In the present study, the utility of FTA cards was assessed for sample collection for the countrywide molecular epidemiological study of leishmaniasis in Peru.

MATERIALS AND METHODS

Study areas and sample collection. The study areas were located in tropical rainforest at an altitude of less than 500 m above sea level (a.s.l.), in higher forest at 500 to 1,000 m a.s.l., and in the Andean highlands and in inter-Andean valleys at 1,000 to 3,000 m a.s.l. The samples were taken from patients who were assessed at the rural health centers of the 15 departments of Peru, viz., Amazonas, Ancash, Aprimac, Ayacucho, Cajamarca, Cusco, Junin, La Libertad, Lambayeque, Lima, Loreto, Madre de Dios, Pasco, Piura, and San Martin (Fig. 1). The patients visited their health centers for the diagnosis and treatment of leishmaniasis, having routine dermatological, parasitological, and epidemiological examinations. The present patients and/or their parents voluntarily consented to participate in this study, under the approval of health centers of the Ministry of Health at each department, for the FTA card-collecting procedures employed. All the referred patients from whom the present FTA card materials were obtained were systemically treated for 20 to 30 days with 10 to 20 mg/kg body weight/day of pentavalent antimony. In the case of an incomplete clinical cure of the lesions, another cycle of treatment was given according to the recommendation of the World Health Organization. The clinical controls and treatment protocols were exclusively conducted by local physicians at each rural health center of the departments mentioned above. Clinical samples were collected from 132 patients with cutaneous (CL), mucosal (ML), and mucocutaneous leishmaniasis (MCL) by local physicians and well-trained laboratory technicians at the routine diagnosis of leishmaniasis in each rural health center of the departments mentioned above. Tissue materials were taken by aspirating or scraping the margins of active lesions of a patient and then spotted onto an FTA classic card (Whatman, Newton Center, MA) and stored at room temperature. Six to 18 months after sample collection, 2-mm-diameter disks were punched out from each filter paper and washed three times with FTA purification reagent (Whatman) and once with Tris-EDTA buffer. The disks were air dried and subjected directly to PCR amplification.

Identification of Leishmania species. Leishmania species were identified from cytochrome b (cyt b) gene sequences (9, 13). PCR amplification with a pair of specific primers, L-cyt-AS (5’-GCGGAGAGRAGAAAGGGC-3’) and L-cyt-AR (5’-CCACTCATATAATCTATA-3’), was performed with 30 cycles of denaturation (95°C for 1 min), annealing (55°C for 1 min), and polymerization (72°C for 1 min) using Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan). One microliter of the PCR product was reamplified with L-cyt-S (5’-GGGTAGGTGTTTTGAGG-3’) and L-cyt-R (5’-CTCAATAAACAAATCTAATATATCAATT-3’). The products were cloned into the pGEM-T Easy vector system (Promega, Madison, WI), and sequences were determined by the dyelex chain termination method using a BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA).

L. (V.) braziliensis was differentiated from L. (V.) peruviana by a PCR-restriction fragment length polymorphism (RFLP) analysis of the mannose phosphate isomerase (MPI) gene as described previously (9).

RESULTS

Clinical samples from 132 patients with leishmaniasis living in various areas of endemicity in Peru were collected with the cooperation of local physicians and well-trained laboratory technicians. The samples, aspirated or scraped from patients’ lesions and directly spotted onto FTA cards, were subjected to PCR targeting the cyt b gene. The amplification was repeated not more than twice for samples negative in the first PCR in order to obtain gene fragments of the parasites. Leishmanial cyt b gene fragments were obtained from 81 patients (31 females and 50 males, ranging in age from 3 months to 75 years) out of the 132 sampled (61%). Parasite species were identified by analyzing the cyt b gene sequences and PCR-RFLP results for MPI genes (9). The distribution of Leishmania species by department of Peru is presented in Fig. 1. In this study, the utility of FTA cards was assessed for sample collection for the countrywide molecular epidemiological study of leishmaniasis in Peru.

Among the 81 samples, the three most prevalent species were L. (V.) peruviana (61.7%), L. (V.) braziliensis (22.2%), and L. (V.) guyanensis (12.3%), results which mostly correspond to previous findings (1, 12). In the areas facing the Pacific Ocean, such as Lima, Ancash, La Libertad, Lambayeque, and Piura, most of which are part of the Andean highlands, all the cases were identified as L. (V.) peruviana infections (Fig. 1). Additionally, L. (V.) peruviana was identified in the highland areas of Amazonas, Apurimac, Cajamarca, and San Martin. L. (V.) braziliensis was present in Amazonas, Ayacucho, Cajamarca, Cusco, Junin, Loreto, and San Martin, and L. (V.) guyanensis in Amazonas, Junin, Pasco, and San Martin (Fig. 1). No hybrid species [L. (V.) peruviana/L. (V.) braziliensis] was detected based on the MPI gene analysis. L. (V.) lainsoni was detected in a patient from Madre de Dios by the sequencing and phylogenetic analysis of cyt b (Fig. 1). Furthermore, L. (V.) shawi, never before reported in Peru, was identified in patients from Madre de Dios and Junin based on cyt b gene analyses (Fig. 1). When the distribution of Leishmania species by ecological region was assessed, L. (V.) peruviana was identified in Andean highland areas, whereas the other four species, L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) lainsoni, and L. (V.) shawi, were mainly distributed in rainforest regions (Table 1). Of the latter, L. (V.) guyanensis was mostly identified in lower-altitude rainforest regions (Table 1).

Among all cases examined, children less than 10 years old were affected more (38.3%) than the other age groups (11 to 20 years, 17%; 21 to 30 years, 15%; 31 to 40 years, 11%; 41 to 50 years, 3%; and over 50 years, 4%). Most patients had typical ulcerative cutaneous lesions (80/81, 98.8%) but a very small number had mucosal (2/81, 2.5%) and mucocutaneous (1/81, 1.2%) lesions. No diffuse cutaneous lesion was observed. The number of cutaneous lesions per patient ranged from 1 to 6, and the diameter of lesions ranged from 2 to 60 mm, independent of the Leishmania species. No characteristic differences in cutaneous lesions among causative Leishmania species were observed. Of the 81 patients, three had lesions on nasal mucosa. Of the three, one from Junin was clinically diagnosed with mucocutaneous leishmaniasis (MCL) based on clinical course, and the parasites isolated from the mucocutaneous lesion were identified as L. (V.) braziliensis. In the two other cases, one each from Ayacucho and Apurimac, the mucosal lesions seemed to have disseminated from cutaneous lesions on the face, suggesting cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML), not MCL. The parasites isolated from these cutaneous lesions were identified as L. (V.) braziliensis and L. (V.) peruviana, respectively.

DISCUSSION

In this study, FTA cards were used for less invasive sampling as part of an extensive epidemiological study of leishmaniasis in Peru. Of the 132 patients sampled, parasites were successfully identified in 81 patients from 15 regions of the country based on analyses of the cyt b and MPI genes. Although the detection efficiency was lower than originally expected, the results provide further insight into the geographic distribution...
of *Leishmania* species. Notably, the present study, for the first time, showed that *L. (V.) shawi* is associated with cases of CL in Peru.

There is no longer any doubt about the diagnostic value of molecular biological methods for leishmaniasis. Several PCR-based methods have been developed and widely used for the detection and identification of parasite species in patient specimens (7, 16, 21). As a source of DNA material, biopsy samples
from cutaneous lesions are used in many cases of CL; however, less invasive methods of collecting samples have been devised, such as the scraping and aspirating of cutaneous lesions and exudation with cotton swabs and filter paper (2, 5, 8, 14, 15). All these methods require the extraction and purification of DNA for PCR analysis. With FTA technology, these processes can be skipped since the samples spotted onto the FTA card are readily lysed and the nucleic acids are captured on the card. Furthermore, there is almost no risk of contamination among samples, unlike dried or fixed materials on filter paper. Another great advantage for epidemiological research is that the cards facilitate the collection and storage of samples. This makes it easier to gain the cooperation of local physicians and other medical and paramedical personnel in areas of endemicity, leading to the efficient collection of specimens/materials from different areas, including remote locations. Furthermore, sampling directly from the patients’ lesions eliminates (i) the possibility of contamination and/or mixing of Leishmania isolates during short- or long-term passages of culture in the laboratory and (ii) the pain and suffering caused by a skin biopsy. In this study, the parasite was detected and identified in 81 of 132 specimens, which was a lower efficiency than originally expected. However, no comparison of the detection rates of parasites among different diagnostic methods was made, since the present trial was focused on the collection of data on the countrywide distribution of Leishmania species using easy and less invasive methods. The successful detection of parasites using the present FTA cards was considered to depend largely on the sample collection process performed by individual medical personnel at different institutions; for example, all samples tested positive in one area, whereas no parasites could be detected in any of 12 samples from another area. Different from extracted DNA samples, parasite DNA is not evenly spread in the FTA card but localized in areas where parasites in the tissue materials are fixed. Thus, the areas of the card containing parasites must be punched out for successful detection. Diluted samples, such as blood-containing materials, reduce the concentration of parasites in the cards, resulting in a decrease in the detection efficiency. In addition, sampling must be performed more carefully when an infection of parasites of the subgenus Leishmania (Viannia) is suspected, since the number of these parasites in cutaneous lesions is generally much lower than the number of parasites of the subgenus Leishmania (Leishmania) in lesions. For proper sample collection using an FTA card, it is recommended that tissue materials be taken by aspirating or scraping the margin of active lesions of a patient, avoiding contamination with blood as much as possible, and then be spotted onto a limited area of an FTA card. Although FTA cards are useful for the purpose of diagnosing leishmaniasis in individual patients, the collection method is considered to be more appropriate for a massive and countrywide ecological study to determine the prevalent causative parasite species in each area where leishmaniasis is endemic; this tool, therefore, would also be useful in the disease control and surveillance procedures/phases.

In a previous study, Leishmania species in Peru were investigated by MLEE, and the 5 prevalent species, L. (V.) braziliensis, L. (V.) peruviana, L. (V.) guyanensis, L. (V.) lainsoni, and L. (L.) amazonensis, were defined, of which the first three were predominant (12). Another extensive study performed using multilocus PCR-RFLP identified the distribution of L. (V.) lainsoni, Leishmania (L.) mexicana, and a putative hybrid, L. (V.) braziliensis/L. (V.) peruviana, in addition to the three predominant species (1). The geographic distribution of the predominant species defined in the present study corresponded overall to the results of the previous studies; L. (V.) braziliensis was mostly found in the tropical rainforest, L. (V.) peruviana in Andean highland areas, and L. (V.) guyanensis in northern and central rainforest regions (1, 12). Endemicity largely depends on the fauna that host the vector sand fly species and is probably affected by factors like environmental changes. Therefore, continuous countrywide investigations of both sand flies and Leishmania species will be necessary. In addition, influences on the distribution of Leishmania species from population movements should be taken into consideration.

The present study showed, for the first time, the occurrence of L. (V.) shawi in Peru. L. (V.) shawi was originally isolated from monkeys, sloths, coatis, and the sand fly Lutzomyia whitmani from primary forest in the northern part of Brazil (11). Although information on this species is limited, the infection of humans has been recorded in the Atlantic rainforest regions of northern and northeastern Brazil (3, 18). In the present study, two cases of L. (V.) shawi infection were identified, the first report of L. (V.) shawi outside Brazil. Both patients were from lower rainforest regions of Peru, one from the border with Bolivia and northwestern Brazil and the other from the central region of Peru. Both these areas are more than 2,000 km from regions of endemicity in Brazil (3, 18), indicating that the distribution of L. (V.) shawi could be much more extensive than previously thought. Lutzomyia whitmani is a suspected vector of L. (V.) shawi in Brazil and has been recorded in Peru (22). Thus, Lutzomyia whitmani may be associated with the transmission of L. (V.) shawi in Peru. Finally, the present study demonstrated the usability of FTA cards in sample collection for the ecological study of leishmaniasis. The results provide further insights into the geographic distribution of Leishmania species in Peru.

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REFERENCES


17. Reference deleted.

18. Reference deleted.
