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Identification of *Leishmania* spp. by Radiorespirometry

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SYNOPSIS. Preliminary investigation of the application of radiorespirometric technic to protozoan parasites of man indicates a potential for rapid identification. This technic, developed for identification of bacteria, was modified for use with culture forms of *Leishmania*. Five strains of *Leishmania* were compared: 2 of *L. donovani*, 2S and K; *L. brasiliensis*, 2936 and B; and 1 of *L. tropica*, A. Consistent and rapid (~2 hr) identification was obtained by the radiorespirometric procedure. A computer-type analysis of the radiorespirometric profiles of the 5 strains permitted correct identification of each isolate at the strain level 100% of the time. This technic offers several advantages over many current procedures for identification of protozoan parasites: (A) It is simple, rapid and highly reproducible. (B) Since it does not rely on visual or spectrophotometric determination, it may be conducted in the presence of optically complex substances. (C) It requires relatively low numbers of organisms ($\sim 2 \times 10^5$/^{14}C-labeled substrate). (D) It is based on differential enzymic activity between species and strains of organisms and therefore, ultimately, on inherent genetic determinates of the parasites. (E) Further development of the procedure and accumulation of a data reference "bank" would allow automation of most of the identification process.

Index Key Words: *Leishmania donovani*; *Leishmania brasiliensis*; *Leishmania tropica*; promastigotes; radiorespirometry; identification.

PARASITES of the genus *Leishmania* present serious clinical problems because of the absence of a reliable, sensitive, and rapid means of identification of nosodemes and evaluation of chemotherapeutic compounds. A prerequisite to drug treatment, distinguishing of *Leishmania* species is still based upon the organotropism of the parasite in man and the epidemiologic features of the infection (24, 28, 45). Neither clinical pathology nor epidemiology is a reliable diagnostic parameter, however, since *Leishmania* species in early infections and following drug treatment do not always occupy typical loci of infection (12, 17, 24, 25, 35), and species overlap in their geographic distribution (2). More modern protozoologic methods for parasite identification are based on serologic technics, study of parasite fine structure, refractivity of a series of laboratory animals to the protozoon in question, isoenzyme distinctions, polypeptide analysis, and DNA buoyant density profiles (2, 4-7, 9, 11, 16, 30, 31, 36, 38, 39, 47, 48). None of these methods is well suited for large-scale clinical identification and rapid drug-sensitivity screening.

The study reported here was designed to investigate the application of a new bacteriologic technic to the diagnosis of human leishmaniasis. Microbial radiorespirometry was first reported by Levin et al. (20) as a new approach to the selective detection of coliform bacteria in water. Levin and his co-workers further developed this method for water quality evaluation and for use in the space probes, notably the Viking Mission to Mars, as an experiment to detect extraterrestrial life forms (18, 19, 21, 22). There have been numerous reports on detection and enumeration of bacteria by radiorespirometry (for pertinent references, see Ref. 41). Schrot et al. (41) reported detection in both blood and pure culture of fewer than 100 colony-forming units (CFU) bacteria in less than 2 hr using radiorespirometry. Subsequently, the application of this procedure to identification of bacteria was attempted by Schrot and his co-workers (40, 42). Based upon computer-assisted probability analysis of radiorespirometric profiles alone, the bacterial isolates were identified to species 92% of the time. Antibiotic sensitivity determinations by radiorespirometry have a high level of agreement (greater than 90%) with standard methods. The radiorespirometric principle of identification is based on slight differences in activity or lack of homologous enzymes and, therefore, ultimately on inherent genetic determinates of the parasite species or strain involved.

In the present preliminary study we tried only to determine whether Schrot's radiorespirometric method would be useful in distinguishing the 3 clinically recognizable forms of human leishmaniasis: (a) visceral or kala azar, caused by *Leishmania donovani*; (b) cutaneous or Oriental sore, caused by *Leishmania tropica*; and (c) mucocutaneous or espundia, caused by *Leishmania brasiliensis*.

MATERIALS AND METHODS

Strains of *Leishmania* used in this study were obtained from Dr. Robert Herman, Rutgers University, and Dr. Franklin Neva, Laboratory of Parasitic Diseases, National Institutes of Health. The 5 strains were *Leishmania donovani* strain 2S (Sudan-Malakal), *Leishmania donovani* strain K (Khartoum), *Leishmania tropica* strain A (from the Middle East), *Leishmania brasiliensis* strain 2936 (from Panama), and *Leishmania brasiliensis* strain B (from Brazil). Henceforth, the respective capital letter or number after the species name will be used to designate each strain. All these organisms were grown in diphasic blood-agar medium, used for maintenance of stock cultures of *Leishmania* at N.I.H. This medium is a slight modification of Tobie's medium (44). Stock cultures were maintained in either N.I.H. or Tanabe's medium (8).

To prepare cultures for radiorespirometry, 25 ml N.I.H. medium base were added to a 250-ml deLong culture flask and 15 ml Locke's solution (pH 7.1) added immediately before use. The inocula contained $\sim 5 \times 10^4$ promastigotes from 7-day stock cultures incubated at 25 C. Experimental cultures were maintained at 25 C and organisms harvested during late logarithmic growth (4-5 days postinoculation). Radiorespirometric rate dropped sharply in stationary phase cultures. Therefore, growth was monitored to avoid variation caused by culture growth phase. Before being used for radiorespirometry, the cultures were examined for possible contaminants by microscopic examination and by standard bacteriologic procedures on blood agar plates. The protozoa were washed 3× in sterile Locke's solution by centrifugation at ~755 *g* at 5 C for 12 min. Under the conditions employed, it was necessary to remove the nutrient medium before incubation with ^{14}C-labeled substrates to prevent masking of the metabolic profile. After the final wash, the pellet was resuspended in sterile Locke's without dextrose (pH 7.2). The concentration of the organisms was determined by hemacyto-

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TABLE 1. *Typical raw data and calculations for* Leishmania donovani *K replicate #7506222.**

Substrate	Non-biologic control cpm	Replicate #7506222 cpm	Replicate minus 15 cpm background	Control minus 15 cpm background	Replicate minus control	Replicate corrected for mach. efficiency dpm	Probability values from Table 2
Urea	18	25	10	3	7	44	0.56
Glycine	18	131	116	3	113	706	0.22
Alanine	14	121	106	0	106	663	0.22
Serine	13	1,328	1,313	0	1,313	8,206	0.67
Valine	19	226	211	4	207	1,294	0.33
Leucine	12	4,250	4,235	0	4,235	26,469	1.0
Aspartate	15	6,716	6,701	0	6,701	41,881	1.0
Ornithine	12	734	719	0	719	4,494	1.0
Lysine	9	29	14	0	14	88	0.56
Arginine	16	24	9	1	8	50	0.67
Histidine	31	105	90	16	74	463	0.33
Phenylalanine	20	139	124	5	119	744	0.78
Tyrosine	15	82	67	0	67	419	1.0
Tryptophane	30	24	9	15	0	0	0.33
Glucose	20	6,981	6,966	5	6,961	43,506	1.0
Sucrose	19	3,089	3,074	4	3,070	19,188	1.0
Maltose	13	57	42	0	42	263	1.0
Xylose	18	358	343	3	340	2,125	0.67
Mannose	82	6,316	6,301	67	6,234	38,963	1.0
Trehalose	21	237	222	6	216	1,350	1.0
Sorbitol	19	64	49	4	45	281	1.0
Erythritol	14	35	20	0	20	125	0.67
Mannitol	13	602	587	0	587	3,669	1.0
Formate	17	32	17	2	15	94	0.56
Acetate	26	1,866	1,851	11	1,840	11,500	0.33
Propionate	27	60	45	12	33	206	0.44
Lactate	54	733	718	39	679	4,244	0.78
Succinate	15	272	257	0	257	1,606	0.89
Malonate	16	32	17	1	16	100	0.89
Citrate	18	29	14	3	11	69	0.67
Malate	20	102	87	5	82	513	0.56
Gluconate	20	29	14	5	9	56	0.78
						Product probability score = 1.1×10^{-6}	

* On this date background measured 15 cpm and machine efficiency measured 16%.

sents the data base for an individual strain. Identification of an unknown strain was obtained by comparing the results of a radiorespirometric test (e.g. column 6, Table 1, for replicate #7506222) to all the probability frequency charts available for known species or strains. The probability that the unknown strain could be identical to a known strain was computed by multiplying the probabilities for the 32 individual substrates. $P_P = P_1 \times P_2 \times P_3 \times \ldots P_{32}$, where P_P = the product probability score, and $P_1, P_2, P_3, \ldots P_{32}$ = probabilities on the chart corresponding to the dpm value ranges resulting from the test organism's profile. A sample calculation is illustrated in column 7, Table 1, for the replicate (column 6, Table 1) #7506222 of *L. donovani* K. To calculate P_1, for the substrate urea, the dpm value is equal to 44. This dpm value falls within the dpm value range on Table 2 of $x < 50$, so that the P_1 value for urea (indicated by an asterisk) equals 0.56, and this value is then recorded in column 7, Table 1. To calculate the product probability, P_P, of the 32 probability scores, the values in column 7, Table 1 are multiplied by one another until all 32 numbers have been included, i.e. $0.56 \times 0.22 \times 0.22 \times \ldots 0.78 = 1.1 \times 10^{-6}$. Identification of an organism is based upon the probability frequency chart from which the highest product probability, P_P, was obtained. Blank spaces were filled with probability values of 0.01 to avoid introduction of 0 values into the calculation of P_P. (For example, when the *L. donovani* K replicate #7506222 was compared with all the data for *L. donovani* K (Table 2), the product probability score that this test organism was the K strain equalled 1.1×10^{-6}. Product probability scores obtained when this replicate #7506222 was matched against non-homologous strain data bases are shown in Table 3 (numbers marked with *). When this replicate #7506222 was compared to the entire data base for *L. donovani* 2S, the P_P value was 6.1×10^{-16}. These P_P values were for *L. tropica* A, 5.2×10^{-13}; for *L. brasiliensis* 2936, 3.8×10^{-13}; and for *L. brasiliensis* B data, 3.9×10^{-13}. In view of these results, replicate #7506222 would have to be identified as a *L. donovani* strain K. The statistical method employed was similar to that described by Friedman & MacLowry (10) and Robertson & MacLowry (34).

This method was tested by comparing each of the 29 replicate profiles to the probability frequency chart of each strain. Because data for the replicate being tested was not withdrawn from the data base the charts were termed uncensored. Uncensored charts were used because in this preliminary investigation the entire data base was not large enough to permit removal of the test replicates. A larger data base may have increased the sensitivity of the method by reducing any tendency toward false positive or negative results caused by the use of uncensored data.

In 29/29 (100%) trials the correct match was made, and the test replicate profile was grouped with the data base of the known strain to which it belonged. Replicates #7506132 and #7506133 of *L. brasiliensis* 2936 had P_P values of 1.6×10^{-8} and 7.1×10^{-7}, respectively, when matched with their own data base (Table 3), but P_P values of 9.4×10^{-8} and 6.4×10^{-8},

TABLE 3. *Product probability scores for the 5 strains of* Leishmania.

Leishmania tropica A	*Leishmania tropica* A	*Leishmania donovani* 2S	*Leishmania donovani* K	*Leishmania brasiliensis* 2936	*Leishmania brasiliensis* B
7505301	4.7×10^{-8}	9.7×10^{-13}	4.8×10^{-14}	1.6×10^{-14}	3.3×10^{-17}
7505302	1.4×10^{-5}	4.5×10^{-16}	1.0×10^{-12}	4.9×10^{-14}	2.1×10^{-15}
7506021	9.3×10^{-7}	1.4×10^{-13}	3.7×10^{-11}	1.1×10^{-13}	1.0×10^{-14}
7506901	1.5×10^{-6}	2.6×10^{-14}	2.5×10^{-16}	1.7×10^{-14}	2.3×10^{-18}
7506092	2.2×10^{-5}	8.9×10^{-13}	1.3×10^{-13}	2.8×10^{-15}	6.1×10^{-18}
7506093	8.1×10^{-7}	1.8×10^{-15}	2.1×10^{-14}	2.3×10^{-16}	3.4×10^{-19}
Mean	6.5×10^{-6}	3.4×10^{-13}	6.4×10^{-12}	3.3×10^{-14}	2.0×10^{-15}
Leishmania donovani 2S					
7505301	1.5×10^{-16}	4.0×10^{-9}	6.3×10^{-22}	1.8×10^{-16}	1.8×10^{-21}
7505302	1.2×10^{-12}	4.3×10^{-7}	3.5×10^{-13}	4.7×10^{-16}	1.3×10^{-17}
7505303	6.9×10^{-13}	3.1×10^{-6}	7.5×10^{-15}	9.7×10^{-14}	4.6×10^{-17}
7506091	2.5×10^{-13}	6.9×10^{-6}	6.5×10^{-15}	1.5×10^{-14}	9.1×10^{-19}
7506101	6.8×10^{-16}	5.8×10^{-7}	4.5×10^{-18}	1.0×10^{-16}	3.8×10^{-20}
Mean	4.3×10^{-13}	2.2×10^{-6}	7.3×10^{-14}	2.3×10^{-14}	1.2×10^{-17}
Leishmania donovani K					
7505121	9.9×10^{-18}	1.7×10^{-21}	2.9×10^{-6}	2.8×10^{-14}	6.1×10^{-12}
7505191	1.6×10^{-14}	2.6×10^{-18}	1.5×10^{-5}	2.2×10^{-13}	1.4×10^{-10}
7505192	2.2×10^{-14}	7.5×10^{-21}	3.2×10^{-7}	1.4×10^{-14}	2.0×10^{-14}
7505231	6.7×10^{-14}	1.8×10^{-18}	1.9×10^{-5}	8.4×10^{-14}	1.5×10^{-12}
7505232	2.9×10^{-12}	9.7×10^{-17}	6.0×10^{-5}	6.6×10^{-13}	4.6×10^{-11}
7506221	1.1×10^{-15}	5.9×10^{-15}	3.6×10^{-9}	1.5×10^{-14}	9.2×10^{-15}
7506222	*5.2×10^{-13}	*6.1×10^{-16}	*1.1×10^{-6}	*3.8×10^{-13}	*3.9×10^{-13}
7506301	2.2×10^{-11}	3.2×10^{-9}	9.7×10^{-9}	1.0×10^{-12}	5.1×10^{-16}
7506302	2.9×10^{-9}	4.8×10^{-11}	1.5×10^{-6}	3.3×10^{-13}	2.0×10^{-15}
Mean	3.3×10^{-10}	3.6×10^{-10}	1.1×10^{-5}	3.0×10^{-13}	2.2×10^{-11}
Leishmania brasiliensis 2936					
7505131	2.1×10^{-17}	2.7×10^{-15}	9.5×10^{-15}	1.3×10^{-12}	4.9×10^{-18}
7505231	2.5×10^{-18}	4.5×10^{-30}	6.3×10^{-22}	7.3×10^{-15}	1.2×10^{-22}
7506131	1.4×10^{-16}	5.6×10^{-30}	3.6×10^{-13}	4.7×10^{-8}	3.2×10^{-12}
7506132	3.5×10^{-13}	2.0×10^{-17}	2.2×10^{-13}	1.6×10^{-8}	9.4×10^{-8}
7506133	7.5×10^{-15}	6.5×10^{-16}	7.7×10^{-11}	1.1×10^{-7}	6.4×10^{-8}
Mean	7.2×10^{-14}	6.7×10^{-16}	1.6×10^{-11}	3.5×10^{-8}	3.2×10^{-8}
Leishmania brasiliensis B					
7505121	2.3×10^{-18}	5.1×10^{-19}	1.2×10^{-11}	4.3×10^{-11}	5.2×10^{-4}
7505231	4.9×10^{-19}	9.7×10^{-18}	3.2×10^{-13}	3.8×10^{-14}	3.2×10^{-6}
7506131	3.5×10^{-16}	8.3×10^{-19}	2.9×10^{-13}	2.6×10^{-9}	2.9×10^{-5}
7506132	1.9×10^{-17}	3.4×10^{-19}	1.0×10^{-12}	3.5×10^{-9}	5.8×10^{-5}
Mean	9.3×10^{-17}	2.8×10^{-18}	3.4×10^{-12}	1.5×10^{-9}	1.5×10^{-4}

* P_P values for the replicate #7506222 of *L. donovani* K when matched with probability frequency charts for homologous and heterologous strain data.

For taxonomic purposes, serologic methods have been combined with numerical analysis. Matossian-Rogers et al. (27) subjected 4 species of *Leishmania* to many immunologic tests including both homologous and heterologous antisera in agglutination, fluorescent antibody, and passive cutaneous anaphylaxis reactions. Serologic analyses of *Leishmania* species, however, have always been difficult because of the high degree of cross-reactivity among the species (6). To avoid this problem, Zuckerman and her co-workers tried serotype grouping of *Leishmania* spp. based upon reactions of promastigote antisera with metabolic by-products, Excreted Factors. These reactions reflected regional variants among *Leishmania* spp., but were not species-specific (38, 39, 48). Saf'janova & Avakjan (36) reported that they had achieved species identification of *Leishmania* using ferritin-labeled antigen-antibody localization by electron microscopy. It was shown also that serologic differences among *Leishmania* spp. could be demonstrated by various methods (3-5, 23, 33).

Serologic and metabolic differences among *Leishmania* spp. attest to the fact that there are genetic differences among these species. It was on this premise that Chance et al. (7) approached the *Leishmania* identification problem by analysis of DNA buoyant density profiles. This technic, however, as the others cited above, has some disadvantages when simplicity and rapid diagnosis is required for use in a clinical laboratory. Consequently, identification of *Leishmania* spp. is still based on clinical findings, pathologic findings and epidemiology.

The present radiorespirometric study approached the problem of identification of *Leishmania* at the species level by differential utilization of organic substrates. Metabolic differences appear to

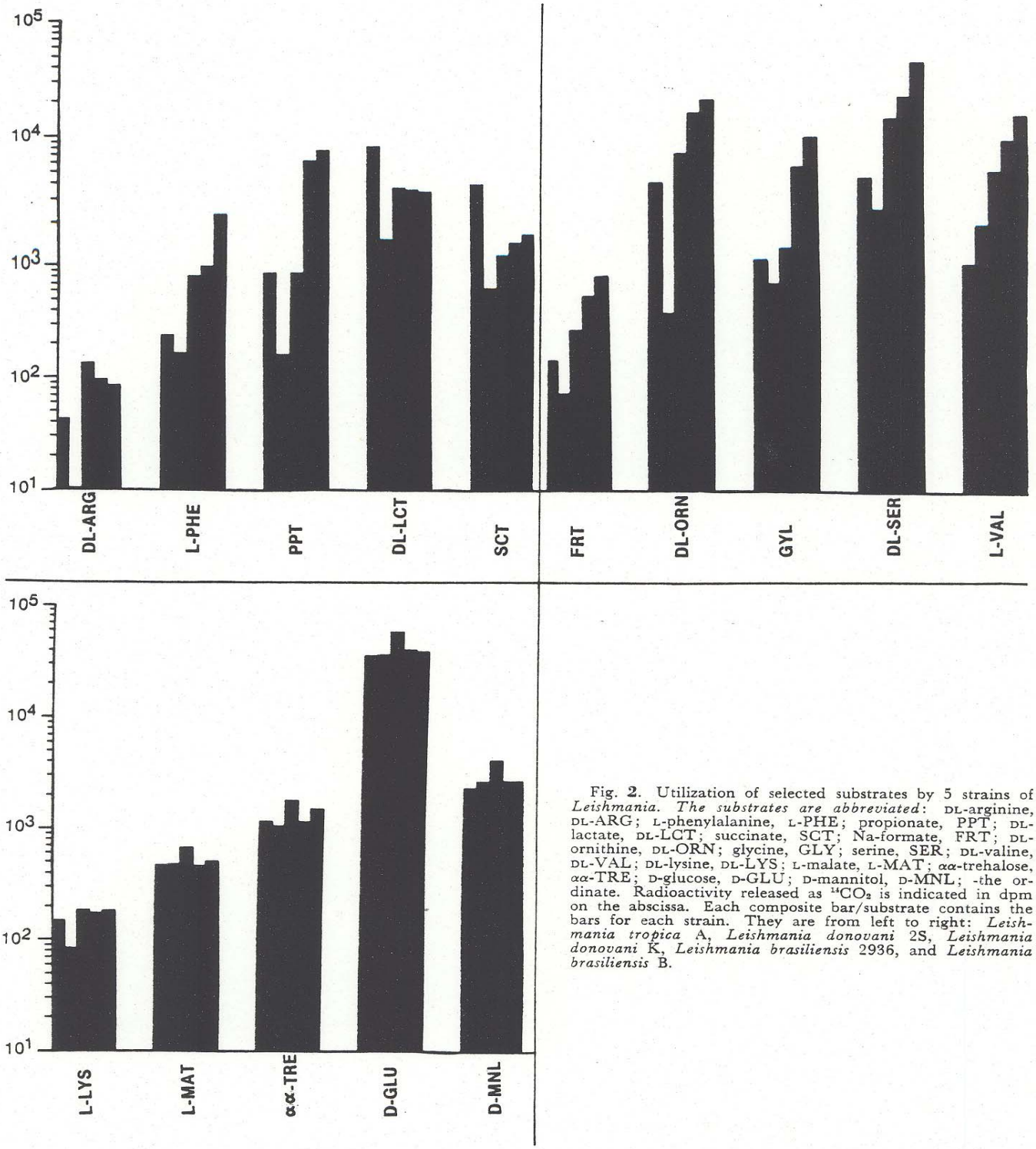

Fig. 2. Utilization of selected substrates by 5 strains of *Leishmania*. *The substrates are abbreviated*: DL-arginine, DL-ARG; L-phenylalanine, L-PHE; propionate, PPT; DL-lactate, DL-LCT; succinate, SCT; Na-formate, FRT; DL-ornithine, DL-ORN; glycine, GLY; serine, SER; DL-valine, DL-VAL; DL-lysine, DL-LYS; L-malate, L-MAT; αα-trehalose, αα-TRE; D-glucose, D-GLU; D-mannitol, D-MNL; -the ordinate. Radioactivity released as $^{14}CO_2$ is indicated in dpm on the abscissa. Each composite bar/substrate contains the bars for each strain. They are from left to right: *Leishmania tropica* A, *Leishmania donovani* 2S, *Leishmania donovani* K, *Leishmania brasiliensis* 2936, and *Leishmania brasiliensis* B.

ERRATA

1. Page 463, Synopsis, line 6 change "100%" to "97%".

2. Page 465, first line of last paragraph, change "29/29 (100%)" to "28/29 (97%)".

3. Page 468, first column, sixth line from bottom, change "100%" to "97%".

4. Page 468, second column, eleventh line from bottom, change "100% (29/29)" to "97% (28/29)".

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