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Method Article

Chemotaxis in *Leishmania (Viannia) braziliensis*: Evaluation by the two-chamber capillary assay

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A B S T R A C T

Chemotactic responses play a significant role during *Leishmania (V.) braziliensis* differentiation through its life cycle and during infection. The aim of this description has been to portray the modified "two-chamber capillary chemotaxis assay" as a technique useful for quantitative *in vitro* evaluation of *Leishmania* chemotaxis after reviewing the methods described until now to assess chemotaxis *in vitro* in *Leishmania sp*. This valued simple and reproducible method convenient for parasite migration determination, was tested by the use of controlled changes in monosaccharide (D-glucose and D-fructose) concentrations as referent ligands. The validation of the method demonstrates that this technique is useful to evaluate the relationship existing between parasite migration towards the monosaccharides and sugar concentration. This means that within specific ranges, parasites attracted by the monosaccharide migrate towards more concentrated solutions and accumulate (higher number of parasites) at that spot. Interestingly, both the time course of the experiment and the osmolality of the solution influence parasite migration capacity. Our validation suggests that this improved methodology quantitatively evaluates taxis of *Leishmania* towards/against different substances. On the basis of our herein presented data, we conclude that this technique is a novel, rapid and reliable screening method to evaluate chemotaxis in *Leishmania*.

- The two-chamber capillary chemotaxis assay was standardized for *Leishmania*.
- The technique is useful to quantitatively evaluate in vitro chemotaxis in *Leishmania*.
- Parasite migration was characterized by monosaccharide chemical gradients.
- This assay is a novel, rapid and reliable screening method to evaluate chemotaxis.

Contain between 1 and 3 bullet points highlighting the customization rather than the steps of the procedure.

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Resource availability:	*If applicable, include links to resources necessary to reproduce the method (*e.g. *data, software, hardware, reagent).*

Method details *[Methodological protocols should be in sufficient detail to be replicated. There is no word limit! You can include figures, tables, videos – anything that you feel will help others to reproduce the method. The main focus of the paper should be on the technical steps required for this method, more than results; where appropriate, guide the reader through the procedure and provide all extra observations or "tricks" alongside the protocol. Results and Discussion are not sections included in the MethodsX format. However, providing data that validate the method is valuable and required. This section could become a "method validation" paragraph within the Method Details section.]*

Materials and methods

Materials

D-glucose and D-fructose, as well as the analytical grade materials used to grow the cells and perform the biological experiments, were purchased From Sigma-Aldrich Co (St. Louis, MS, USA).

Strain and culture conditions

The reference strain *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/LTB 300) was kindly provided by Dra. Noris Rodríguez, (Instituto de Biomedicina, Universidad Central de Venezuela). *Leishmania* promastigotes were grown at 26 °C in semisolid blood agar supplemented with glucose-NaCl medium (glucose 1.5%, NaCl 0.85%, w: v). Promastigotes at their late log growth phase were collected by centrifugation at 1 500 x *g* for 10 min at room temperature (RT). The use of late log growth phase parasites is essential to ensure a minimum percentage of dividing cells within the parasite population. The medium was decanted and the cells were softly resuspended in buffer A: Hepes, 10 mM pH 7.3; NaCl, 132 mM; KCl, 3.5 mM; $CaCl_2$, 1 mM and $MgCl_2$, 0.5 mM. The cells were centrifuged again and the buffer was discarded; the cells were resuspended in buffer A up to the desired cell density. Buffer A (osmolality = 288 mOsm kg^{-1}) was used in all experiments as the solution to test chemotaxis of untreated parasites. When used for substances other than monosaccharides, for example neuropeptides, a buffer slightly different, Buffer A1 (NaCl 135 mM; KCl 5 mM; $CaCl_2$ 1 mM; $MgSO_4$ 1 mM; Hepes 20 mM; 292 mOsm/Kg, pH 7.4) is used. Buffer A1 is convenient to preserve the integrity of used peptides without affecting the overall standardized conditions of the experiment.

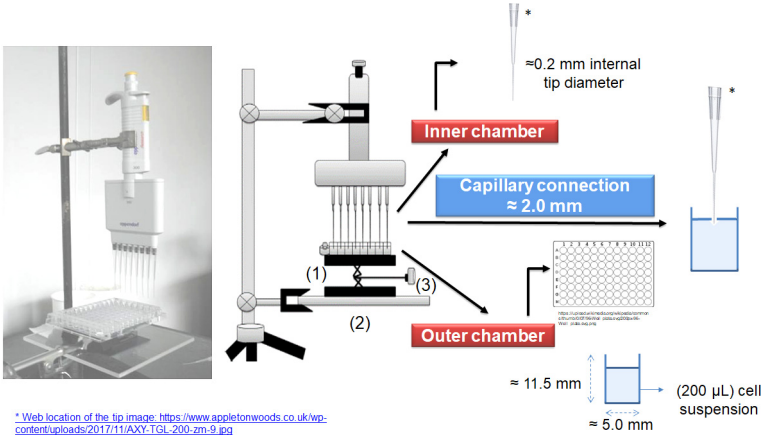

Fig. 1. Setup of capillary chemotaxis assay with a multichannel pipette. To ensure capillary communication between the inner and outer chambers, long gel electrophoresis loading tips, with an internal diameter -at the tip- of ≈0.2 mm were used. A thorough leveling of the system was performed by the use of an (1) adjustable stand located over an (2) acrylic base and coupled to a (3) micrometer. The tips (inner chamber) were filled with the experimental substance (untreated, positive control or increasing concentrations of carbohydrates). The wells (outer chamber) were filled with the *L.* (*V.*). *braziliensis* suspension. The two chambers are connected only via (3) capillary entries of the tips containing the test substances. The distance between the tip and the cell suspension must be ≈ 2.0 mm.

Carbohydrate solutions

The D-glucose (100 mM) and D-fructose (300 mM) stock solutions were prepared in buffer A and kept frozen at −20 °C until use. Dilutions of carbohydrates were freshly prepared for each experiment in buffer A.

Two-chamber capillary assay

The chemotactic response of *Leishmania* promastigotes was measured by the two-chamber capillary assay initially developed by Köhidai [12] and modified by us. In our set up (Fig. 1), the tips of an 8- or 12-channel-micropipette were used as the inner chamber and the wells of a sterile 96-well plate were used as outer chamber of the two-chamber system. To ensure capillary communication between the inner and outer chambers, long gel electrophoresis loading tips, with an internal diameter of 1 mm or less were used. A thorough leveling of the system was performed by the use of an adjustable stand located over an acrylic base and coupled to a micrometer. The tips were filled with the experimental substance (untreated, positive control or increasing concentrations of carbohydrates). The wells were filled with 200 µl of the *L.* (*V.*). *braziliensis* suspension. The micrometer was then used to approximate the multiwell plate (outer chamber) to the pipette multichannel (inner plate) to reach a distance between outer and inner chambers of 2 mm. The system was then incubated for various time periods. At the end of the incubation time the solution from inner chambers (containing the cells that migrated) was transferred to clean wells of the multiwell plate. Then the cells that migrated into the inner chamber were fixed in formaldehyde (2%) diluted (if necessary, 1:10) in PBS (phosphate buffer 0.05 M, pH 7.2; NaCl 0.9 M). The cells were then directly enumerated in a Neubauer chamber or hemocytometer.

A detailed description of the experimental procedure is included in Box 1. It is important to emphasize that these experiments do not require special equipments. The minimum requirement include: a 96-well plate, an 8- or 12-channel micropipette, an adjustable stage, a light microscope, a Neubauer chamber or hemocytometer. All these implements are normally present in each cell biology laboratory. This statement certifies this method as a low-cost technique.

Box 1. Experimental Procedure: Two chamber capillary assay for Leishmania.

1. Let *Leishmania* promastigotes grow at 26 °C in semisolid blood agar supplemented with glucose-NaCl medium (glucose 1.5%, NaCl 0.85%, w: v) until their late log growth phase.
2. Collect the cells by centrifugation at 1 500 x *g* for 10 min at room temperature (RT).
3. Decant the medium and softly resuspend the cells in buffer A: Hepes, 10 mM pH 7.3; NaCl, 132 mM; KCl, 3.5 mM; $CaCl_2$, 1 mM and $MgCl_2$, 0.5 mM (osmolality = 288 mOsm kg^{-1}). Repeat this procedure once more. Resuspend the cells in buffer A up to the desired cell density.
4. Have the D-glucose (100 mM) and D-fructose (300 mM) stock solutions ready to be used. They should be prepared in buffer A and be maintained frozen at −20 °C until use. Prepare fresh dilutions of carbohydrates for each experiment in buffer A.
5. Transfer the cells (200 µl of a 4 × 10^7 cell ml $^{-1}$ suspension) to the corresponding wells of a sterile 96-well plate. These wells will be used as outer chambers of the two-chamber system.
6. Fill long gel electrophoresis loading tips of an 8- or 12-channel-micropipette with the desired probe solutions (100 µl). Each tip will function as the inner chamber of the two-chamber system. To do so, pour 200 µl of the desired solutions in clean and sterile wells of a 96-well plate and suck the desired volume of each solution with the multichannel pipette. The tips are filled -each- with the corresponding experimental substance (untreated, positive control or increasing concentrations of carbohydrates).
7. Locate the 96-well plate over the adjustable stand placed over an acrylic base, coupled to a micrometer.
8. Attach the filled 8- or 12-channel-micropipette to the stand by a fixed clamp.
9. Be sure that the leveling of the stand is correct by the use of a horizontal leveling.
10. Turn the micrometer clockwise to approximate the multi-well plate (outer chamber) to the multichannel pipette (inner plate) until reaching a distance between outer and inner chambers of 2 mm.
11. Incubate the system for the desired time period.
12. Turn the micrometer counterclockwise to separate the multi-well plate from the multichannel pipette.
13. Transfer the suspension from each inner chamber (containing the cells that migrated) to clean wells of the multi-well plate.
14. Fix the cells in formaldehyde (2%) dilute (if necessary, 1:10) in PBS (phosphate buffer 0.05 M, pH 7.2; NaCl 0.9 M).
15. Enumerate the cells in a Neubauer chamber or hemocytometer.

Standardization and quality control parameters

In order to guarantee the reproducibility, quality and validity of the two-chamber capillary assay system as a good model for chemotaxis evaluation four parameters were standardized. A thorough discussion of the validation is given in this article.

1. Optimal cell density in the outer chamber: The optimal cell density (4 x 10^7 cell ml^{-1}) was chosen from the literature following previously established parameters [13,17].
2. Optimal incubation time: To choose the optimal incubation time for the assays, the chemotactic response was evaluated at 15, 30, 45 and 60 min.
3. Monosaccharide concentrations: The monosaccharide concentration test-points at each tip were chosen according to previously established data [17]. D-Glucose from 4 to 100 mM; D-Fructose from 4 to 225 mM. Each tip contained a different fixed concentration ensuring the stability of the setpoint monosaccharide concentration.
4. Solution's osmolality: The solution's osmolality (mOsm kg^{-1}) was measured with a single sampler osmometer (The Advanced TM Osmometer Model 3D3; Advanced Instruments, INC. USA), with a linear reproducibility (0 to 400 mOsm kg^{-1}) of 2% (1 S.D.). All solutions used in the study were evaluated by this method.

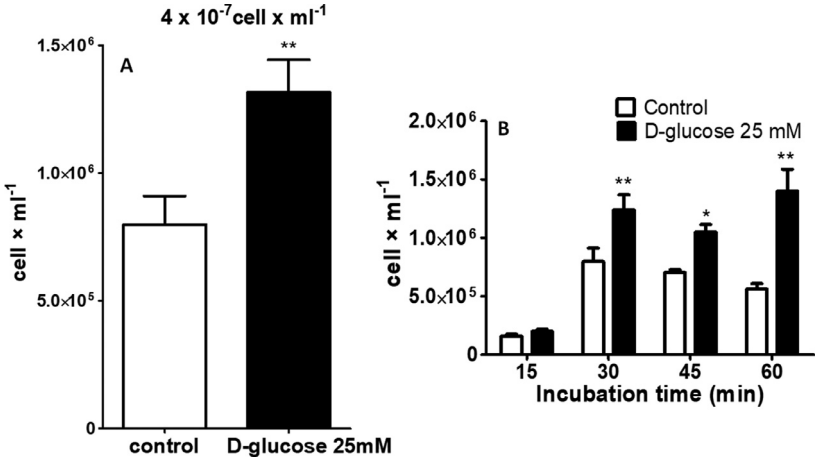

Fig. 2. Effect of (A) cell density on the chemotactic response elicited by 25 mM glucose in *L.* (*V.*) *braziliensis* promastigotes, (B) time course of chemotaxis elicited by 25 mM glucose in *L.* (*V.*) *braziliensis* promastigotes. The data were analyzed using Student's *t*-test. Values represent mean ± SEM and levels of significance are shown in the figure as $^{*}p<0.05$; $^{**}p<0.01$.

Analysis of results

Data describing the number of cells migrating into the inner chamber were expressed as mean values ± standard error of the mean (SEM) of at least five experiments. Differences between carbohydrate treated and untreated cells were tested for statistical significance with Student's *t*-test with the aid of the GraphPad program (5th version).

Method validation

Specific chemical stimuli play a role in *Leishmania* parasite migration and host-parasite interaction, and motility is a fundamental functional response to those chemical stimuli. Quantitative study of chemotaxis allows a holistic comprehension on how specific chemicals act as chemoattractant/chemorepellents and to make simple methods reliable they should be thoroughly standardized. In an effort to standardize the two-chamber capillary method herein we present results of fundamental parameters required to evaluate migratory responses, in order to understand the behavior of *Leishmania* parasites in the presence of chemoattractant compounds.

Cell density

Initially and to standardize the two-chamber capillary assay we selected two cell densities (4 × 107 ml-1 and 8 × 107 cell ml^{-1}) and assayed the chemotactic properties of 25 mM D-glucose vs. buffer A (control). Our results demonstrated that 4 × 10^7 cell ml^{-1} were sufficient to obtain chemotactic responses around 200% ($p = 0.004$) compared to buffer A (control) (Fig. 2A). Therefore, we used this cell density to perform successive experiments.

Incubation time

To further standardize the two-chamber capillary assay we evaluated the chemotactic response at various incubation times (Fig. 2B). The optimal response was obtained when using 30 min ($p = 0.0045$) (Fig. 2B). Shorter time periods (15 min) were not sufficient to induce significant chemotactic responses, while longer incubation times (45 and 60 min) resulted in a significant number of cells in the inner chamber ($p = 0.001$). The monosaccharide concentrations in the tips

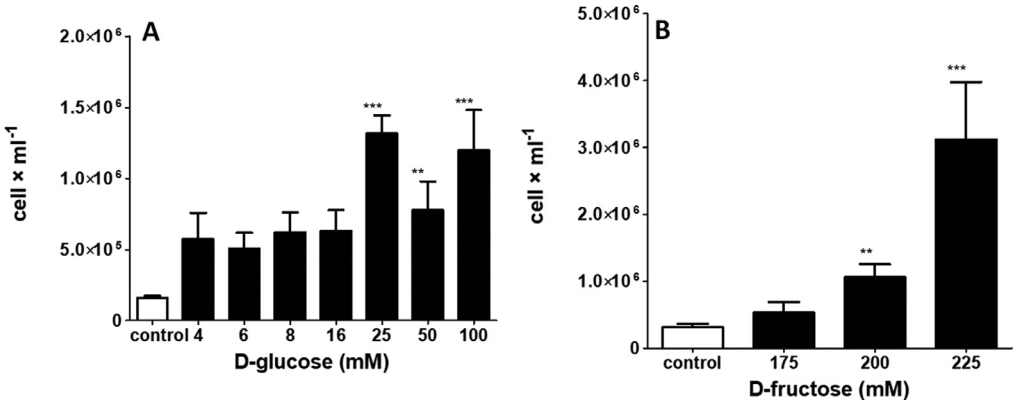

Fig. 3. Concentration course study of chemotaxis elicited by (A) D-glucose, (B) D-fructose. The data were analyzed using Student's *t*-test. Values represent mean ± SEM and levels of significance are shown in figure as $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.0001$.

would also be perturbed and could have influenced the observed response. Since our aim was to measure exclusively chemotactic and not chemokinetic responses, the stability of the setpoint monosaccharide concentration at each tip was fundamental for our analysis. This matter was certain when using incubation times of 30 min. Additionally, a short 30 min incubation is a convenient timing. Although synchronized cultures were not been used for the herein presented experiments, we have used late log growth phase parasites. Dividing times in *Leishmania* parasites are circa 18 h. Thus, 30 min incubation times would not make dividing cells to interfere with results of migrating cells, the timing selected for the rest of the experiments presented.

Effect of monosaccharide concentrations

To validate our system we analyzed the chemotactic response of *L.* (*V.*) *braziliensis* promastigotes with increasing concentrations of D-glucose and D-fructose. The results are summarized in Fig. 3. The experiments performed with D-glucose demonstrated that this monosaccharide promotes parasite migration at 25, 50 and 100 mM (25 mM = 50 mM <100 mM) compared to untreated cells (Fig. 3A). Lower concentrations than 25 mM (D-glucose 0 - 16 mM) did not promote chemoattractant responses in *L.* (*V.*) *braziliensis*. Likewise, D-fructose induced significant chemoattractant responses but only at a higher concentration range (200 and 225 mM, Fig. 3B). Lower concentrations (< 175 mM) of D-fructose also elicited migratory responses; however, they were not statistically significant.

Effect of osmolality

To further validate our system and to discriminate if osmolality influences the migration of the cells, we evaluated the osmolality of the solutions used in the herein presented data and compared the chemotactic activity (% number of migration of cells respect to untreated vs. osmotic pressure) at the concentrations of carbohydrates used.

As expected, the osmolality of carbohydrate solutions increased proportionally with increased solution concentration (Tables 1 and 2). The response of *L.* (*V.*) *braziliensis* promastigotes to osmotic gradients was different for each tested carbohydrate. In the presence of D-glucose, cell migration was significant when the osmolality increased at least to 22 mOsm kg^{-1} compared with buffer A (Table 1), while for D-fructose migration occurred when the osmolarity increased at least to 212 mOsm Kg^{-1} compared to buffer A (Table 2). A thorough debate of these results follows.

Table 1
Comparative outline of D-glucose parameters (concentration, chemotactic activity, osmotic pressure) on *L.* (*V.*) *braziliensis* promastigotes.

D-Glucose (mM)	Chemotactic activity[a] % migrating cell number (control=100%)	Osmotic pressure[b] mOsm kg^{-1}
Control	100.00 + 0.00	0 + 1.1
4	122.00 + 20.28	3.00 + 0.47
6	124.24 + 15.43	5.67 + 0.82
8	151.14 + 19.17	7.67 + 1.41
16	158.07 + 25.93	16.34 + 1.25
25	269.00 + 62.62*	22.00 + 0.47
50	183.22 + 21.62*	48.00 + 1.25
100	293.57 + 45.47*	89.00 + 0.47

[a] Values (mean ± SEM) given as the percentage of cells that migrate relative to control (n = 5–8).
[b] Values (mean ± SD) given as the increasing mOsm kg^{-1} relative to control (n = 3).
* $p < 0.05$.

Table 2
Comparative outline of D-fructose parameters (concentration, chemotactic activity, osmotic pressure) on *L. (V.) braziliensis promastigotes*.

D-Fructose (mM)	Chemotactic activity [a] % cell number migrated (control=100%)	Osmotic pressure [b]D mOsm/kg
Control	100.00 + 0.00	0.00 + 1.10
175	178.70 + 34.50	184.00 + 0.47
200	231.57 + 81.85*	212.66 + 2.05
225	448.31 + 97.29**	242.00 + 1.70

[a] Values (mean ± SEM) given as the percentage of cells that migrate relative to control (n = 5–8).
[b] Values (mean ± SD) given as the increasing mOsm kg^{-1} relative to control (n = 3).
* $p < 0.05$.
** $P = 0.01$.

Development of chemotactic assays in *Leishmania*, why we need a different one

Chemotaxis is an essential response present in all phyla. For example, unicellular microorganisms actively search the optimal environment which provides elements for nourishment, proliferation and survival. Microbes analyze their surroundings and move towards the attractive chemical substances, and away from repellent, toxic compounds. Chemotaxis is not the only migratory response at hand in microorganisms. Microorganisms also react to changes in hydrostatic pressure, light, magnetic fields, osmotic pressure, temperature, etc.

Surface membrane receptors play significant roles in chemotactic signaling. However, signaling mechanisms (a network of Che-proteins or G-protein coupled receptors) and effector systems (prokaryotic flagellum or cilia, flagellar or ameboid movement) differ in prokaryotic and eukaryotic cells. In prokaryotic cells (i.e., bacteria) the organism functions as a “point sensor” due to its small size compared to the gradient. These microorganisms cannot distinguish differences in chemical concentrations between their two axial poles; therefore, their migratory behavior is composed of two phases: linear swimming paths and tumbling. On the contrary, eukaryotic cells with a bigger size, distinguish differences in chemical concentrations along their axial poles.

However, both types of organisms express a variety of surface membrane receptors and intracellular signaling pathways that allow the cell to detect chemical concentration changes even in quiescent phases. All this means that understanding chemotaxis is essential for the comprehension of the behavior and physiology of motile cells. Chemotaxis is a fundamental step in the successful

interaction between host and parasite, as chemical signals are involved in their mutual recognition and in the migratory responses that determine infection [18].

Protozoan parasites of the genus *Leishmania* cause a disease with symptoms ranging from self-healing cutaneous lesions to non-healing mucocutaneous and visceral ailments that affect approximately 30 million people in Africa, the Middle East, and Central and South America [18,23]. The parasite life cycle includes two hosts and two morphologically distinct forms: the flagellated promastigote, living within a sand-fly (Phlebotominae subfamily), and the non-flagellated amastigote infecting mammals including humans.

In the sand-fly digestive tract, the promastigote reacts to changes in concentrations of nutrients and chemicals due to the exhaustion of the previous blood meal; the pathogen migrates towards the proboscis and simultaneously differentiates into an infective cell. The parasite enters the host skin after the female sand-fly sucks/bites the mammal searching for the blood it needs for eggs maturation. Afterwards, the parasite and the host immune cells recognize each other, migrate, and respond functionally, the parasite infecting the immune cell and the latter phagocytizing the invading agent. This process marks the onset of the disease in the host [11], making it clear that chemotactic behavior is a fundamental response throughout the *Leishmania* life cycle. Once inside the host cell, for example, the macrophage, *Leishmania* (motile) promastigote differentiates into the non-motile amastigote form. As the amastigote multiply, host-cell membrane explodes and amastigotes infect additional macrophages spreading out the infection.

Methods to analyze the chemotactic response of *Leishmania* (summarized in Table 1) were initially developed by R.S. Bray [2]. In this method, a promastigote suspension is introduced into a chamber made of a sawn-off disposable 1-ml tuberculin syringe covered with a 1.2 µm-pore-size Millipore filter. This apparatus is immersed in a putative chemotactic fluid; the attractive capacity of the substance is evaluated after an incubation period, by counting the parasites in the chemotactic fluid.

Oliveira et al. recognized this method as insensitive, as it did not offer a stable setting away from external influence, and additionally it required high concentrations of attractant for chemotaxis to be detected; thus, they developed an assay based on Adler's 1973 bacterial chemotactic method. In the Oliveira et al. [17] method a gradient of the substance to be assayed is build up inside a capillary tube: the capillary tubes filled with the defined media called *washing and incubating solution* [WIS plus enriched bovine serum albumin (0.004%)] contains the test chemotactic agent and agarose (0.2%). Before the solution solidifies, modeling clay is used to push the solution 1 cm inside the end of the tube and covered with Parafilm©. The gradient is formed as the 1 cm unfilled end is filled with WIS. Afterwards, this tube is submerged within the promastigote suspension. After an incubation period, the capacity of the substance to promote migration is evaluated by counting the parasites inside the capillary tube. The drawback of this procedure is that no control vs. chemotactic compounds is included thus suggesting the potential presence of osmotactic movements in the overall chemotactic result [13].

Leslie et al. [13] modified the original Oliveira's method. In this modified method the capillary tube is filled with the same buffer used to prepare the promastigote suspension and is set vertically in a "bijou" tube. The open end of each capillary is then immersed in the promastigote suspension. After an incubation period, the migrating cells are collected from the 1 cm open-end and counted with a hemocytometer [13]. This improved method that used higher promastigote densities, and includes control tubes without chemotactic agents, permitted the discrimination between osmotactic and chemotactic responses in *Leishmania*.

Further modifications were introduced by Barros et al.. In this method, the mean time that the promastigotes remain moving in a straight line (without abrupt changes of direction) is measured in the absence or presence of homogenous concentrations of the agent that promotes taxis. Although this method was developed to distinguish chemotactic from osmotactic responses, the results can only be used to make conclusions on the length of time it takes for 'adaptation' [15] -a form of memory that allows detection, by temporal sensing mechanisms, of changes in the environment- to occur within a new environment of different concentration of testing chemotactic agents.

Finally, recent studies used optical tweezers to quantify chemotaxis in *Leishmania* (*L.*) *amazonensis*. In this method, beads trapped by an optical tweezer function as a transducer for measuring the propulsion force induced by flagellar motion. Of note, with this method, Pozzo et al. [18] quantified

the strength and directionality of the flagellar force. This method allowed to dissect that *Leishmania* uses circular movements and tumbling, while bacteria uses straight-line movement and tumbling, to detect gradients. This means that *Leishmania* has a clear sense of direction and moves with great force.

Herein, we modified the "two-chamber capillary assay" initially developed by Köhidai [12] and applied it to study chemotaxis in *Leishmania* promastigotes. This method was originally used to measure chemotaxis in *Tetrahymena pyriformis*. Our aim has been to validate this technique for *Leishmania* and to standardize the most important parameters needed to quantify migratory responses. Two hexoses (D-glucose and D-fructose) that were previously found to be attractive to promastigotes [9] were used as reference ligands. We selected these two ligands since carbohydrates are a major energy source for promastigotes in vitro and a major component of the sand fly diet and elicit chemotactic responses. Our results suggest that this improved methodology quantitatively evaluates the taxis of *Leishmania* towards/against gradients of different substances. On the basis of our herein presented data we also conclude that this novel, rapid and reliable technique has the additional advantage of the use of multichannel micropipettes to provide high accuracy regarding the precision of the range of concentrations tested. Thus, it is a screening method to evaluate chemotaxis of *Leishmania* that may be helpful also in diagnosis and drug development. A preliminary report of this extended method manuscript has been published in Spanish [6].

Debate on the herein presented validation

Many studies describe chemotaxis and osmotaxis as processes carried out by cells and microorganisms (Blair, 1999, [20], Barros et al. [1]). The capillary assay, first developed by Pfeffer [19] and later improved by Adler (1973), is the most commonly used quantitative method. Our work is one of the contemporary proposals in this area. Herein we combine two techniques - the two-chamber and the capillary assay – a modification initially used by Köhidai [12] to measure chemotaxis in *Tetrahymena pyriformis*. We describe the standardization and validation of the method for its use in *Leishmania* parasites. Our results suggest that the best parameters to measure chemotaxis in *Leishmania* with the herein presented method are a cell density of 4×10^7 cell ml^{-1} washed promastigotes in the outer chamber and 30 min incubation time.

Safar et al. [21] described for Tetrahymena that 15–20 min was their optimal incubation time. For *Leishmania* the optimal timing herein described was 30 min. This 10 min difference might be related to cell model specificities between *Tetrahymena* and *Leishmania*. A short 30 min incubation is a convenient timing. Synchronized cultures have not been used for the herein presented experiments; however, we have used late log growth phase parasite. As the dividing time of *Leishmania* parasites is circa 18 h, a 30 min incubation time would not make dividing cells to interfere with the results of migrating cells presented herein.

To validate the method, two carbohydrates were used as chemotactic agents. D-glucose increased the migration and was chemoattractant to the parasites at 25 mM. The highest concentration (100 mM) tested herein, also increased the migratory response but this behavior might be due instead to osmotactic attraction [13], or a combination of chemotactic and osmotactic responses (Table 1). In contrast, D-fructose increased cell migration only at higher concentrations (175, 200 and 225 mM) than glucose, significantly at 200 and 225 mM; the effect at D-fructose 175 mM was not significant although the osmolality was 184 mOsm kg^{-1} higher than that of buffer A (Table 2). These results suggest that *L.* (*V.*) *braziliensis* needs much lower concentrations of D-glucose than of D-fructose to migrate and therefore is more sensitive to D-glucose than to D-fructose (Fig. 3). *L.* (*V.*) *braziliensis* promastigotes increased their migratory response when there was a difference in the osmolarity of the solutions. However, a difference of 22 mOsm kg^{-1} was enough for D-glucose solutions to trigger migration, whereas a difference of 212 mOsm kg^{-1} was needed for D-fructose solutions to trigger the same effect. These results suggest that *L.* (*V.*) *braziliensis* requires a much lower osmotic gradient of D-glucose than of D-fructose to migrate and therefore has significantly higher osmotactic sensitivity to D-glucose than to D-fructose (Tables 1 and 2).

Altogether the results suggest that responses at 100 mM D-glucose (89 mOsm kg^{-1}) are not (only) osmotactic, as has been proposed by Leslie et al. [13]. This is especially highlighted by the fact that 100 mM D-fructose (105 mOsm kg^{-1}) (data not shown) did not elicit significant parasite

migration. Moreover, in agreement with our findings, the data of Barros et al. (2006) suggest that low (0.001 mM) concentrations of glycine, lactose, mannitol and sucrose elicit chemotaxis. However, the same molar concentrations of NaCl, Hepes or guanosine did not elicit migratory responses. Barros and colleagues argue that if the tactic responses would be osmotactic, as it was proposed by Leslie et al. [13], then NaCl, Hepes and guanosine should elicit migratory responses under the same experimental conditions, which was not the case (Barros et al. [1]). This is especially true for salts like NaCl that dissociate into Na^+ and Cl^- and therefore contribute with two particles instead of one to the osmotic pressure when compared to the non-dissociable substances, e.g. sucrose at the same molar concentration.

The technique used to analyze chemotaxis may also influence the concentration threshold required to elicit the response. Our experiments yield fundamental data to understand chemotaxis and other cell migratory responses since contrary to what Barros et al. (2006) evaluated (the mean time of straight movements), we enumerated the cells that completed the function. That is the number of cells that effectively migrated into the inner chamber. Migration is a complex behavior that needs additional steps that must be included in the response evaluation. Of note, our experiments were conducted in conditions that retain the cellular integrity and do not cause damage to the cells [3,4]. Furthermore, a reduction (but not an increase) in medium osmolality leads to changes in cell shape (shorter) and the release of AAs, mainly alanine, the largest single component of the intracellular pool of free amino acids in both promastigotes and amastigotes [5,16]. Interestingly, mutants lacking LdAAP24 expression, for the specific transport of alanine and proline, are unable to properly respond to an osmotic shock [10]. Additionally, a slight decrease in the growth rate may be caused by an increased average osmolality 308–625 mOsm kg^{-1} [3,4,22] and in response to acute hypertonicity (a rapid increase to 315 mOsm/kg), the Na^+ content of the cytoplasm decreases 33%, vs. control [14]. Finally, the evaluation of migratory behavior and morphological changes produced by aliphatic, monocarboxylic, dicarboxylic, heterocyclic and Sulphur-containing AAs in *L. (L.) amazonensis* and *L. (V.) braziliensis* has demonstrated that L-methionine and L-tryptophan at pM concentrations and L-glutamine and L-glutamic acid at µM concentrations induce positive chemotactic responses, while L-alanine at µM concentrations, L-methionine both at pM and µM concentrations and L-tryptophan, L-glutamine and L-glutamic acid at nM and pM concentrations induce negative chemotactic responses [8].

Since our experiments used solutions with slightly higher osmolality (< 89 mOsm/kg for D-glucose; < 242mOsm/kg for D-fructose) vs. untreated control cells, we would expect that physiological activities like growth or metabolism would not be affected, especially since our experiments only lasted 30 min. D-glucose (25 mM) was the most potent chemoattractant stimulus reported herein, this is probably due to the higher affinity of hypothetic (not yet characterized) D-glucose receptors vs. D-fructose receptors to their respective carbohydrates. The response presented herein and elicited by D-glucose in *Leishmania* suggests that the expression of different receptors with a wide range of affinities might be induced by cellular metabolites ([13,17,1]).

In conclusion, we have standardized a novel method to measure the tactic activity of *L.* (*V.*) *braziliensis* under controlled changes of chemical concentrations of simple substances (e.g. hexoses). When used for different substances like neuropeptides, a buffer slightly different, Buffer A1 (NaCl 135 mM; KCl 5 mM; CaCl2 1 mM; MgSO4 1 mM; Hepes 20 mM; 292 mOsm/Kg, pH 7.4) might be used. Buffer A1 is convenient to preserve the integrity of peptides used without affecting the overall standardized conditions of the experiment.

The two-chamber capillary assay proved to be an effective, reproducible and especially sensible technique for in vitro detection of chemotaxis in *L.* (*V.*) *braziliensis*. The system is extremely useful for quantitative studies of taxis in *Leishmania* and Trypanosomatids under the stress of hydrostatic pressure, osmotic pressure, temperature, etc. As chemotaxis is an essential process of host-parasite interactions, this reliable, reproducible and simple method may also be useful in clinical diagnostics of leishmaniasis, as well as for properties validation of novel, leishmanicidal drugs. For example, we have demonstrated *Leishmania* chemotactic responses to methotrexate conjugates with a terminal serine amino acid (poly-lysine-methotrexate-conjugate), to improve drug-targeting towards the *Leishmania* parasites [7]. On the other hand, it might represent an important tool to understand *Leishmania* migration behavior in the sand-fly, as well as the importance of chemotaxis and other forms of

cell migration for stage development through the parasite life cycle. As previously mentioned, we have demonstrated that during their chemotactic response, *Leishmania* discriminates between slight concentration differences of small and structurally closely related molecules and amino acids. These results suggest that besides their metabolic effects, amino acids play key roles linked to sensory mechanisms that might determine parasite's behavior within the sand-fly [8].

A brief summary of the advantages and limitations of the developed method follows:

1. Cheap and reliable method that may be automated.
2. Being a vertical method, the cells migrate against the ground force making it sensitive.
3. The main communication way between the inner and outer chambers is capillarity thus:
   a. it is a semi closed system where evaporation of fluid is minimized;
   b. there is not diffusion of the chemotactic agent, at least for the first 30 min.
4. Distinguish between chemotaxis and osmotaxis.
5. Morphology of migrating cells can be monitored at the end of the experiment. This might be fundamental if developmental stage experiments are to be done.
6. Useful *in vivo* to evaluate and improve drug-targeting towards *Leishmania* parasites.
7. As it is developed for multichannel pipettes it cannot be used for modeling *in vivo* conditions, in which extracellular matrix components are used to emulate fore xample the skin.
8. If the pipette is not accurately calibrated, the data can give inaccurate results not reflecting the reality of the experiment.

Declaration of Competing Interest

The authors declare that they have no competing interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101223.

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